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REGULAR SURFACE LAYER OF *Azotobacter vinelandii*

by

©

WADE HAMILTON BINGLE

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF MICROBIOLOGY

EDMONTON, ALBERTA

SPRING, 1987

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Date March 19, 1987

ABSTRACT

The regular tetragonal surface(S)-layer of *Azotobacter vinelandii* UW1 could be visualized by freeze-etch electron microscopy but only after the cells were extensively washed with buffer at an elevated temperature. Although the S-layer appeared to be composed of uniform morphological subunits with a center to center spacing of 12.5 nm, image processing of electron micrographs of negatively stained S-layer fragments showed two sorts of tetrameric subunits, differing in apparent mass and interconnected by delicate linkers.

The surface layer, extracted from whole cells or outer membrane fragments with distilled water, produced four protein bands when analyzed by SDS-PAGE, a major 55K band and minor 45K, 30K and 16K protein bands. These minor bands were found to be conformational isomers of the 55K species indicating it was the sole component of the S-layer. This protein, designated the S-protein, was isolated from outer membrane fragments by distilled water extraction and characterized as an acidic (pI 4.4) species possessing no major carbohydrate moiety. S-protein possessed a theoretical monomolecular weight of 60, 218 and an amino acid composition dominated by hydrophobic and acidic amino acids with no detectable cysteine.

Cell surface radioiodination experiments showed that distilled water washing of whole cells extracted essentially all S-protein from the cell surface allowing whole cells to be used as a template for *in vitro* self-assembly of the S-layer. S-protein isolated from whole cells or that isolated from outer membrane fragments, and further purified by gel filtration chromatography as a monomeric species, failed to undergo *in vitro* self-assembly in the presence of a cell wall template. However, S-protein freshly isolated from outer membrane fragments could be induced to self-assemble in the presence of 0.5 mM Ca^{2+}, Mg^{2+} or Sr^{2+}. Analysis of the quaternary structure of freshly isolated S-protein showed it to be composed of a multimeric species with an apparent molecular weight of 255,000. This result in conjunction with the image processing data suggested that S-protein in a tetrameric form, composed the dominant morphological subunit of the array. The tetrameric form of S-protein active in reassembly could be stabilized *in vitro* with 2-5 mM Mg^{2+} or Ca^{2+}, but addition of these species to monomeric S-protein did not result in reformation of the tetramer. Both forms of S-protein possessed a secondary structure dominated by aperiodic and ß-sheet configurations and no alterations in this structure occurred upon oligomerization or in response to divalent cations.

Cells grown in Ca^{2+}-limited medium did not possess a regular S-layer although S-protein was attached to the cell surface in a tetrameric form. Addition of Ca^{2+} or Sr^{2+}, but not more Mg^{2+}, resulted in the reformation of the S-layer. Probes of the gross conformation of surface bound S-protein suggested that Ca^{2+}-mediated crystallization did not require gross changes in the conformation of S-protein. The Ca^{2+} specificity of *in vivo* assembly suggested that *in vitro* reassembly of the S-layer was partly artifactual. Secondly, divalent cation-mediated *in vitro* reassembly did not return the S-layer to the native state because it was sensitive to disruption by monovalent cations at an elevated temperature. These experiments suggested a close association between the S-layer and an outer membrane component *in vivo* which could not be duplicated *in vitro* and that this association influenced the divalent cation selectivity needed for the organization of the array.

PREFACE

Before:

" *I stand in the dark with a blade in my hand, striking at heads.....*"

Dr. Martin Dysart
(from "Equus" by Peter Shaffer)

After:

"This is the even-handed dealing of the world!" he said. "There is nothing on which it is so hard as poverty; and there is nothing it professes to condemn with so much severity as the pursuit of wealth!"

Ebenezer Scrooge
(from "A Christmas Carol" by Charles Dickens)

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This research could not have been accomplished without the help of Dr. R.G.E. Murray and Dr. P.W. Whippey, Departments of Microbiology and Immunology, and Physics, respectively, University of Western Ontario (image processing study) and Dr. C.M. Kay, Department of Biochemistry, University of Alberta (circular dichroism studies). Dr. J.W. Costerton, Department of Biology, University of Calgary, generously provided facilities for freeze-etch electron microscopy. Special recognition is extended to Dr. J.L. Doran; no other individual could have tolerated my company in a 10 ft x 10 ft room for extended periods (weeks) during our infrequent freeze-etch safaris to the University of Calgary. However, I would like to say in my own defence, Dr. Doran possesses certain personal idiosyncrasies himself.

Kim Oikawa and Mike Natriss of the Department of Biochemistry, University of Alberta conducted the circular dichroism and amino acid analysis, respectively. Park Yee of the Department of Soil Science, University of Alberta performed the atomic absorption analyses.

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ABBREVIATIONS

$BBPO_4$	Burk buffer potassium phosphate (4.6 mM K_2HPO_4/1.6 mM KH_2PO_4, pH 7.2 - 7.5)
BSA	bovine serum albumin
EGTA	ethyleneglycol-bis-(ß-aminoethyl ether)N,N'- tetraacetic acid
EDTA	ethylenediamine tetraacetic acid
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethansulfonic acid
KDO	2-keto-3-deoxyoctanoic acid
LPS	lipopolysaccharide
OVA	ovalbumin
Sarkosyl	N-lauroyl sarcosine
SDS	sodium dodecyl (lauryl) sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Triton X-100	octyl phenoxy polyethanoxyethanol
Tris	Tris(hydroxymethyl)aminomethane
OFeBB	iron-limited Burk buffer

1. Introduction

Despite the first identification of regularly arranged protein subunits on the surface of a *Spirillum* species over 30 years ago (Houwink, 1953) and subsequent investigations which showed these structures were common components of prokaryotic cell walls (Koval and Murray, 1986; Sleytr, 1978; Sleytr and Messner, 1983), surface(S)-layers have received relatively little attention. This is probably due to the fact that neither *Escherichia coli* nor *Bacillus subtilis* possess an S-layer, and while widely distributed, only a relative few have been studied in any detail beyond a preliminary examination by electron microscopy.

1.1 Composition of S-layers

Buckmire and Murray (1970, 1973) were the first workers to publish a detailed, rigorous determination of the composition of a bacterial S-layer. Their approach with the *Aquaspirillum serpens* VHA S-layer illustrates a number of principles with respect to establishing the nature of the regularly arranged subunits seen on bacterial cell walls and the importance of electron microscopic observations as a support to biochemical approaches. These workers found that heating whole cells at 60°C for 1h in 1mM $CaCl_2$ caused a disappearance of the hexagonal S-layer from the cell surface when viewed by freeze-etch electron microscopy. Examination of the material released from the whole cells showed the morphological subunits of the array, attached to some sort of membrane-like "backing layer". Although the origin of the backing layer was, and still is, a matter of speculation (Chester and Murray, 1978) it is not integral to the determination of the nature of the regularly arranged subunits. Further treatment of the backing layer and associated subunits with 1.5M guanidine/HCl caused a disappearance of the subunits, leaving the backing layer unaltered. Dialysis of the guanidine/HCl extract and chemical analysis indicated 97.7% protein, 2.3% carbohydrate and undetectable hexosamine, phosphate and ethanolamine, establishing the subunits as proteinaceous; SDS-PAGE revealed that the protein fraction was composed of a single polypeptide of 125-150K apparent molecular weight. Subsequent incubation of solubilized protein with stripped backing layer, in the presence of Ca^{2+}, allowed reassembly of the solubilized protein back into a hexagonal layer indistinguishable from the native layer.

Concurrent and subsequent similar studies with the gram-positive organism *Bacillus* *sphaericus* P-1 confirmed the proteinaceous nature of bacterial S-layers and the indication that they were composed of single polypeptides. A detailed study of the *B. sphaericus* P-1 S-layer was conducted by Henry (1972; Ph.D. Thesis, University of Pittsburgh, Pittsburgh PA) and material from this study appears in a number of review articles (Sleytr, 1978; Sleytr and Messner, 1983). A study of the same S-layer by Howard and Tipper (1973) is more accessible and presumably contains much of the same information. These studies established a number of precedents and solidified a number of speculations concerning S-layers: (1) S-layers are composed of protein subunits, (2) S-layers are composed of single proteins, (3) S-layer subunits are attached noncovalently to other cell wall components including other S-layer subunits and (4) S-layer proteins have the ability to self-assemble *in vitro* if suitable conditions are supplied.

1.2 Fine structure of S-layers

1.2.1 Negative staining and image reconstruction methods

The fine structure of S-layers is determined by examination of negatively stained preparations of S-layer sheets, as much as possible, free of other underlying cell wall components. However, the noise in the micrographs due to heterogeneity in the support surface and variations in staining, limits the ability to observe fine detail. These problems have been overcome by digitizing the electron micrographic image and applying a number of computer-assisted image processing techniques (see Sleytr and Glauert, 1982 for a review of earlier literature). Although the physical and mathematical basis of such methods are beyond the scope of the thesis (and the understanding of the writer), some aspects of these techniques are explored below. All rely upon the repetitive nature of S-layer structure, i.e., they all depend on the image having a regular two-dimensional periodicity so that the multiple copies of the unit cell (the basic repeating unit of the array) can be exactly superimposed. The image processing technique reinforces the signal (periodic part) of the image while the noise (non-periodic part) is averaged out. This translational averaging procedure produces an average of the information present in the image, evening out any random features. Early techniques depended on the use of S-layer fragments with perfect crystallinity (Aebi *et al.*, 1973) because otherwise the various copies of the unit cell would not be exactly superimposed, leading to smearing and loss of detail. Since it is practically impossible to obtain such samples, a newer technique termed correlation averaging was developed (Crowther and Sleytr, 1977; Saxton *et al.*, 1982) which compensates for long and short range distortions in the lattice, so good resolution is no longer so dependent on perfect crystallinity. Another averaging step employed is rotational symmetrization; this procedure exploits the property of rotational symmetry (see below).

While computer-assisted image processing techniques can effectively enhance the structural detail present in electron micrographs, the image ultimately depends on the interaction of the stain with the S-layer. In negative staining, areas of low stain density represent high protein mass areas, while areas of high stain density represent low protein mass regions. Accumulations of stain have been interpreted as pits or pores in the S-layer. Whether or not these regions represent true pores through the layer, or blind pores (pits), can only be inferred by examining both the cell and the environment side of the surface array (Stewart and Beveridge, 1980). The prominence of pores is strongly dependent on the stain level and type (Rasch *et al.*, 1984). Therefore if they appear to be absent it does not necessarily mean they are not present. High stain levels may overwhelm regions of low protein mass giving the impression no protein exists in certain areas (Baumeister *et al.*, 1982) while variable embedding of the S-layer fragment in stain can accentuate different details leading to quite different processed images (Engel *et al.*, 1982).

Despite the impressive technology used to obtain image processed views of S-layers, there can be little solidarity in the interpretation of the resulting image by different investigators. For example, the S-layer of *Sporosarcina ureae* has been imaged by three different laboratories (Baumeister *et al.*, 1981; Burley and Murray, 1983; Stewart and Beveridge, 1980) and compared to the structure of the *B. sphaericus* P-1 S-layer. Baumeister's group (Rasch *et al.*, 1984) considers that the S-layers of *S. ureae* and *B. sphaericus* P-1 are "similar even down to the finest details that can be resolved by electron microscopy; hence it is reasonable to regard them as homologous". On the other hand Stewart and Beveridge (1980) consider these two S-layers to be "quite different".

1.2.2 Some terminology and a classification of S-layer organization

In order to discuss the fine structure of S-layers, it is necessary to consider some terms used in protein crystallography. The smallest unit from which a protein crystal can be built is termed the "asymmetric unit"; for S-layers this is a single polypeptide. "Motifs" are generated using "symmetry operations" performed on the asymmetric unit. The most useful symmetry operation used to describe S-layer structure is rotational symmetry. Rotational symmetry is a "point symmetry operation" and involves the rotation of the asymmetric unit about a fixed point in space which remains unchanged through the rotational operation. If the motif can be generated by six successive 60° turns of the asymmetric unit, hexagonal symmetry is present. Similarly, four successive 90° turns indicates tetragonal symmetry, and three 120° turns indicates trigonal symmetry. The motif which is generated is the actual unit repeated throughout the crystal by translation along the lattice vectors (see below). In practice, a more mathematically defined structure, the "unit cell", which contains the motif, is used to build up the crystal. It is a hexahedron defined in three dimensions by three "lattice vectors" (*a*, *b*, and *c*) which are perpendicular to one another; the crystal is generated by translating the unit cell along these axes. Since S-layers are two-dimensional crystals or three-dimensional crystals one unit cell thick, the third axis *c* is not used and the unit cell can be defined in terms of the vectors *a* and *b* only. The simplest crystals have one motif positioned with the same orientation at each of the four corners of the unit cell. These crystal lattices are called primitive and designated "P". All S-layers examined so far in projection, by image processing techniques, are primitive lattices. The combination of the lattice type (eg. P) with the type of rotational symmetry (eg. P3) is termed the crystal "space group"; in the case of two-dimensional crystals the term "plane group" is also used. It turns out that only plane groups: P1(triclinic), P2 (monoclinic), P3 (trigonal), P4 (tetragonal), and P6 (hexagonal) are possible, and likely, for bacterial S-layers. All such crystals have the protein molecules facing the same way so the cell-side and environment-side of the S-layer are different. This feature is considered necessary due to the differing environments encountered by either side of the S-layer (Baumeister *et al.*, 1986; Sjogren *et al.*, 1985; Stewart *et al.*, 1986).

Baumeister *et al.* (1986) recently proposed a classification scheme, based on all image processing studies to date, outlining the manner in which an interconnected S-layer could be formed from a single asymmetric molecular species. In this scheme, the protein molecules are divided into two regions represented in highly schematic form. The first region is a domain designated "M", where most of the mass is concentrated; this domain is also known as a core region. The other lighter domain, designated "C" (also known as a connectivity region), links neighbouring core regions. These regions are further designated with respect to the crystallographic axes around which they fall. These rotational axes can have nothing to do with the motif or the unit cell, and are evident only after the crystal has been generated by the rotational symmetry operation on the asymmetric unit, creation of the motif and the translation of the unit cell along the lattice vectors to form the crystal. Because eubacterial S-layers are almost exclusively of the P6 or P4 type (Sleytr and Messner, 1983), the following discussion of the organization of S-layers is largely restricted to these structural classes.

1.2.3 Organization of hexagonal S-layers

All hexagonal (P6) S-layers studied so far possess either an M_6C_3 or M_6C_2 organization (Table 1-1; Fig. 1-1). For these types of S-layers, the bulk of the molecular mass is concentrated in a core and a putative pore is usually found at the center; this last feature is not universal (Crowther and Sleytr, 1977). Six spokes (the connectivity regions) emanate from the core; each is considered to be derived from a single polypeptide and the morphological subunit is believed to be a hexamer.

A major difference between hexagonal S-layers of different organisms occurs in the packing of the morphological subunits into the two-dimensional array. The only difference between M_6C_3 and M_6C_2 hexagonal S-layers is in the type of connectivity. Before the publication of Baumeister *et al.* (1986) the individual morphological subunits were considered connected to six other morphological subunits by "linkers". These were classified into two types- "Y" (M_6C_3) and "delta" (M_6C_2) linkers by Stewart and Murray (1982). Although hexagonal S-layers possess a unique six-fold rotational axis they also possess a two-fold and three-fold rotational axis. The type of linker determines whether protein or pore is found at the three-fold or two-fold rotational axes and thus determines the ultimate porosity of the S-layer. One strain of *Bacteriodes buccae* (Table 1-1) appears to possesses a hybrid of the two linkage patterns (Sjogren *et al.*, 1985). While a basic hexameric morphological subunit can be envisioned similar to the S-layers described above, these units have major regions of connectivity at both the two-fold and three-fold rotational axes. Sjogren *et al.* (1985) have proposed that a single protein could give rise to the entire layer, but unfortunately there is yet no biochemical information available on this layer. Interestingly, another strain of *B. buccae* possesses an S-layer of the standard M_6C_3 organization.

1.2.4 Organization of tetragonal S-layers

The organization of tetragonal (P4) S-layers (Table 1-2) is similar to that of hexagonal S-layers (Fig. 1-2). The tetrameric morphological subunit possesses both a core region where most of the protein mass is concentrated, as well as lighter regions of protein mass responsible for the connectivity of the array. According to Baumeister *et al.* (1986) all the spore-forming gram-positive bacteria with tetragonal S-layers appear to be of the same

Table 1-1. Eubacteria possessing hexagonal S-layers which have been studied by image processing techniques.

Species	Classification	Reference
Acetogenium kivui	M_6C_3	Rasch *et al.* (1984)
Aquaspirillum serpens VHA	M_6C_3	Glaeser *et al.* (1979)
Caulobacter crescentus	M_6C_3	Smit *et al.* (1979)
Aquaspirillum serpens MW5*	M_6C_2	Stewart and Murray (1982)
Clostridium thermohydrosulfuricum	M_6C_3	Crowther and Sleytr (1977)
Deinococcus radiodurans	M_6C_2	Saxton and Baumeister (1982)
Synechocystis sp.	M_6C_2	Karlsson *et al.* (1983)
Bacteriodes buccae	$M_6C_{2,3}$	Sjogren *et al.* (1985)

* *A. serpens* MW5 possesses two superimposed S-layers both of the M_6C_2 type.

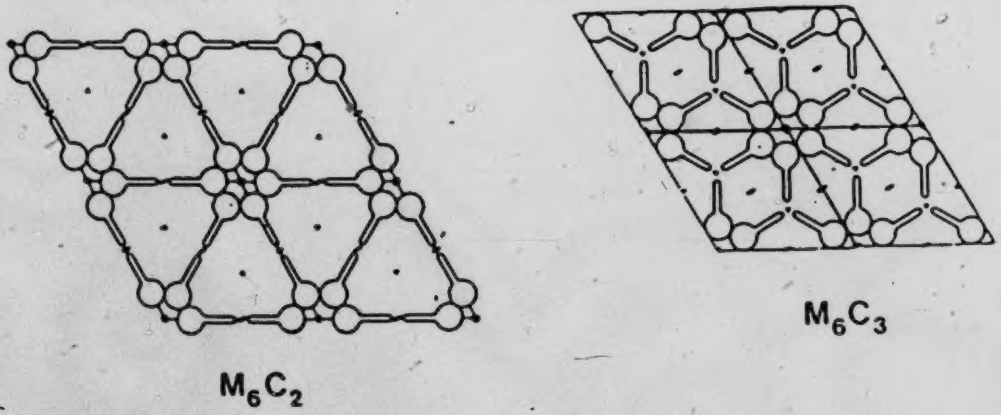

Fig.1-1. Organization of hexagonal (space group, P6) S-layers.

Table 1-2. Eubacteria possessing tetragonal S-layers which have been studied by image processing techniques.

Species	Classification	Reference
Bacillus polymyxa	M_4C_2	Burley and Murray (1983)
Bacillus sphaericus	M_4C_2	Aebi *et al.* (1973) Lepault and Pitt (1984)
Clostridium thermosaccharolyticum	M_4C_2	Crowther and Sleytr (1977)
Sporosarcina ureae	M_4C_2	Stewart and Beveridge (1980)
Aeromonas salmonicida	M_4C_4	Stewart *et al.* (1986)

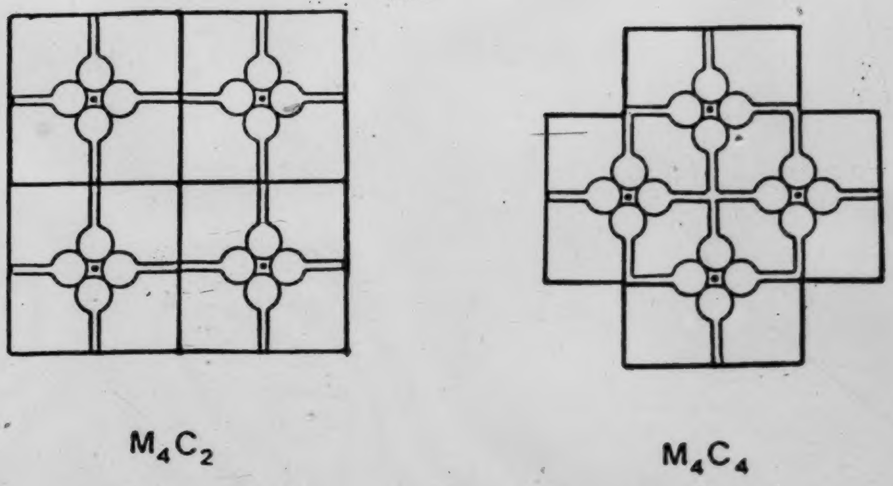

Fig.1-2. Organization of tetragonal (space group, P4) S-layers.

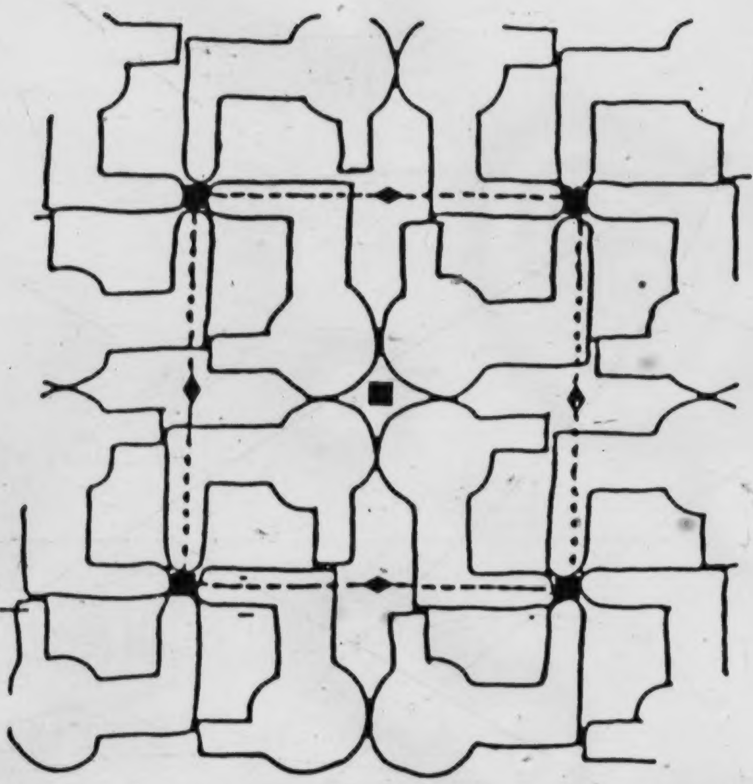

Fig.1-3. A model of the tetragonal S-layers of the spore-forming gram-positive bacteria proposed by Burley and Murray (1983). Reproduced from the Canadian Journal of Microbiology with permission of the National Research Council of Canada, Ottawa. Symbols: ■ 4-fold rotational axes; ◆ 2-fold rotational axes.

type, with major connectivity at the two-fold rotational axis (M_4C_2). Burley and Murray (1983) in another a model of these tetragonal S-layers, included subsidiary connectivity at the other four-fold rotational axis as well (Fig. 1-3).

The only S-layer from a gram-negative organism which has been studied using image processing techniques is that from *Aeromonas salmonicida* (Stewart *et al.*, 1986). The S-layer of this organism, unlike those of the gram-positive species, is of the M_4C_4 type, i.e., major connectivity at the other four-fold rotational axis. In addition, this S-layer can apparently exist in two distinct configurations, a peculiarity which will be discussed later in this thesis.

1.2.5 Organization of trigonal S-layers

Although Baumeister *et al.* (1986) and Sleytr and Messner (1983) have stated that trigonal S-layers have not been described in prokaryotes, it does appear that the so-called hexagonal double S-layer of *Bacillus brevis* 47 could be composed of two proteins each arranged in an S-layer with P3 symmetry (Fig. 1-4). As a supplement to this discovery, an attempt will be made to draw comparisons between the S-layers of *B. brevis* 47 and those of *B. brevis* S1 (Abe *et al.*, 1983), and *Bacillus* CIP 76-111 (Leduc *et al.*, 1973, 1977). These latter organisms may also possess trigonal S-layers. The general view of the cell wall of these organisms appears in Figure 1-5. All the organisms (1) possess protein layers on either side of the peptidoglycan (murein), (2) exhibit a hexagonal array when cell walls are negatively stained and (3) possess two high molecular weight (100-150K) cell wall proteins. Abe *et al.* (1983) consider the amorphous layer to represent one protein and the underlying electron dense layer to represent the hexagonally packed layer because in contrast to negative staining, shadowed cell walls possess no regularly arranged subunits. Abe *et al.* (1983) support this view by examination of a shadowed folded S-layer fragment produced by *in vitro* reassembly of guanidine /HCl extracted cell wall proteins. One side was found to be regularly structured while the other side was unstructured (amorphous). While this conclusion seems attractive there may be another explanation. Intact cells of *Bacillus* CIP 76-111 also possesses an amorphous layer but it is lost during cell wall preparation with no loss of either of the two major cell wall proteins, indicating the amorphous layer is not due to one of these protein species. It is more likely that Abe *et al.* (1983) have not been able to resolve the two closely spaced protein layers in *B. brevis* S1 unlike Yamada *et al.* (1981) for *B. brevis* 47; the amorphous layer commonly seen on the

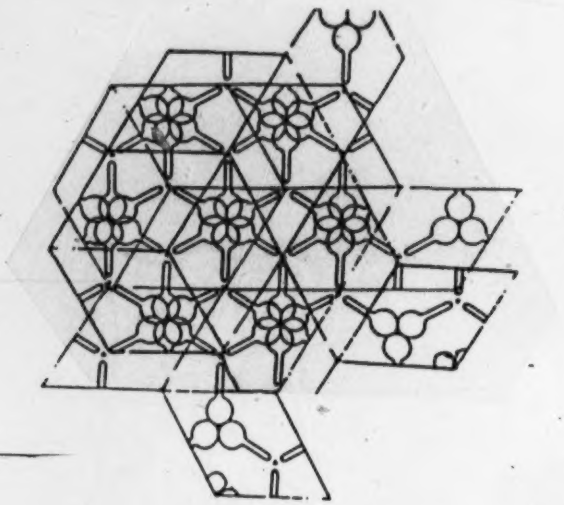

Fig.1-4. Organization of trigonal (space group, P3) S-layers. Overlay the transparency to produce a hexagonal S-layer from two trigonal S-layers when viewed in projection.

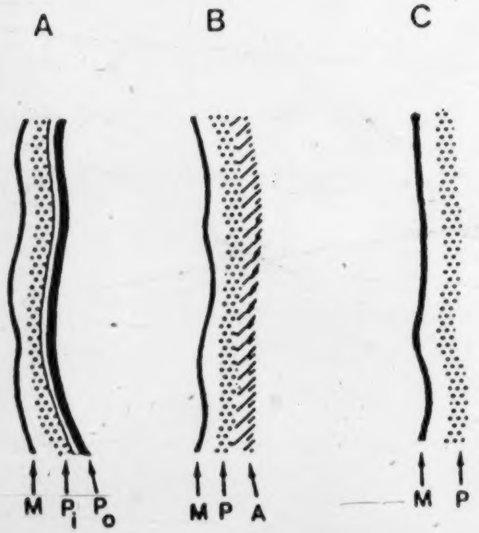

Fig.1-5. Cell wall structure of (A) *B brevis* 47, (B) *B. brevis* S1 and (C) *Bacillus* CIP 76-111. For the sake of clarity, only the S-layers positioned on the outside of the murein (peptidoglycan) have been indicated. The different cell wall layers are not drawn to scale. A, Amorphous layer; M, Murein; P, Protein; P_i and P_o, Inner and Outer protein layers.

cell surface may be due to capsule or adsorbed medium components.

Once the problem of the amorphous layers is discarded, this essentially leaves a similar cell wall structure for all three organisms. A possible explanation is that one protein is on one side of the peptidoglycan and one is on the other. This is unlikely because when the cell wall of *Bacillus* CIP 76-111 is extracted with 6M LiCl, the protein released produces a single band on nondenaturing gels but two bands on SDS or 8M urea gels indicating the two proteins are noncovalently associated in the cell wall. Thus two different proteins form a hexagonal array when viewed in projection. Some light has been shed on the probable structure of these arrays with the isolation of a phage resistant mutant of *B. brevis* 47 (Tsukagoshi *et al.*, 1982). This mutant lacks one protein of the cell wall leaving a lattice with P3 symmetry composed of a single protein lying on the peptidoglycan. Tsukagoshi *et al.* (1982) have presented a number of alternative models of the structure of the wild-type S-layer but a simple explanation is that in the wild type, the other protein also forms a P3 lattice which sits on top of the underlying layer, giving the impression of overall P6 symmetry at first glance (Fig. 1-4). One would expect this should produce an M_6C_3 type of hexagonal S-layer when viewed in projection but the structures of these S-layers have not been resolved to a sufficient level to make out the type of linking unit (Y or delta type). Whether these *Bacillus* species actually do possess two independent S-layers, each with P3 symmetry, awaits further study.

1.3 Purification of S-layer proteins

Assuming S-layers are composed of a single protein species (hereafter referred to as "S-proteins") identification of an extracted protein (judged pure by SDS-PAGE) as an S-layer protein rests on a number of criteria: (1) the candidate protein must be present in the cell wall in sufficient amounts to account for a protein layer which completely envelopes the cell (2) removal of the protein must be accompanied by a loss of the regular pattern from the cell wall when viewed by electron microscopy. But probably the best indication that a protein constitutes an S-layer is (3), the demonstration it can reassemble *in vitro* into an S-layer indistinguishable from the native array on the cell wall. In most cases (see below) when the agent used to disrupt the S-layer is removed, the S-layer will spontaneously reform *in vitro*. This may occur in solution or at a suitable gas /liquid or solid /liquid interface. The most commonly used solid/liquid interface is the 'naked' cell wall fragment from which the protein was released. The appearance of a single band by SDS-PAGE and the ability of the protein to self-assemble *in vitro* leaves no doubt as to its identity. This methodical approach avoids problems which are illustrated by the study of Smit *et al.* (1981) which concerns the S-layer of *Caulobacter crescentus*. These workers recovered S-layer fragments from culture supernatant by centrifugation. Such fragments produced a large number of bands when analyzed by SDS-PAGE leading the authors to suggest that the S-layer of this organism was composed of multiple polypeptides. As pointed out by Koval and Murray (1984) this suggestion is premature and the additional bands could just as easily be due to contaminating outer membrane proteins.

Recent studies involving the isolation of surface array proteins have largely dispensed with the use of whole cells (the approach used by Buckmire and Murray (1970,1973) with *A. serpens* VHA), in favour of the cell wall fraction. After preparation of cell envelopes, the cytoplasmic membrane is usually solubilized with either Triton X-100 (Schnaitman, 1971) or Sarkosyl (Filip *et al.*, 1973) depending on the stability of the surface array to detergents. Subsequent dissociation of the S-layer from the cell wall almost invariably involves the use of urea or guanidine/HCl. The objective of these treatments is to selectively extract the S-layer without coextraction of other cell wall components and most purification protocols do not proceed beyond these single differential extractions. However, it is clear that such preparations of S-proteins are almost never homogeneous as judged by SDS-PAGE.

The studies of Kay *et al.* (1981) and Phipps *et al.* (1983) involving the S-protein of *A. salmonicida* are illustrative of problems encountered with the surface array proteins of gram-negative bacteria. The initial report (Kay *et al.*, 1981) indicated that pure S-protein could be obtained by two extractions of the cell wall with 2% sodium deoxycholate and 6M guanidine/HCl. However, it became clear subsequently, that a major outer membrane protein of 42K could not be reproducibly removed from such preparations. Reliable purification required a DEAE-CL Sepharose chromatographic step (Phipps *et al.*, 1983). Evenberg and Lugtenberg (1982) using a different strain of *A. salmonicida* had to resort to preparative SDS-PAGE in order to purify this S-protein. Similarly, Thornley *et al.* (1974) found that a single extraction of the *Acinetobacter* 199A cell wall with 1M urea released a quantity of an integral outer membrane protein along with surface array protein. The surface array proteins of *A. serpens* VHA (Koval and Murray, 1983) and MW5 (Kist and Murray, 1984; Koval and Murray, 1984) have been extracted from the cell wall with 1-2M guanidine/HCl and 4-6M urea but when analyzed by SDS-PAGE the preparations were not homogeneous (see below).

Although it might be expected that selective extraction of S-proteins would be more successful for gram-positive cell walls, this does not appear to be the case. Most single extraction protocols do not produce homogeneous preparations. S-proteins of the lactobacilli have been extracted from cell wall preparations by Masuda and Kawata (1979, 1981, 1983) using 4M guanidine/HCl. All such preparations contain contaminating polypeptides which range from minor (Masuda and Kawata, 1983) to severe (Masuda and Kawata, 1979). Similarly, while the surface array proteins of *Clostridium thermohydrosulfuricum* and *C. thermosaccharolyticum* can be extracted from the cell wall with 5M the guanidine/HCl and 8M urea, minor bands are always found as contaminants (Sleytr and Thorne, 1976). Attempts have been made to purify the surface array proteins of *B. sphaericus* P-1 (Howard and Tipper, 1973) and 9602 (Hastie and Brinton, 1979a), extracted with 6M urea or 2M guanidine/HCl, by taking advantage of the ability of the protein to reassemble into sheets of tetragonal subunits in solution. However, in neither case was a preparation producing one band on SDS-PAGE obtained. In contrast to all the 'single' S-layers listed above, it appears that the two proteins constituting the double trigonal S-layers of *B. brevis* S1 and *B. brevis* 47 can be released in pure form by a single extraction with 6-8M urea or 2M guanidine/HCl (Abe *et al.*, 1983; Tsuboi *et al.*, 1982).

Some authors have characterized their S-layer protein preparations as pure even though contaminating polypeptides are clearly visible on SDS gels (Howard and Tipper, 1973; Kay *et al.*, 1983). However, as hinted by Koval and Murray (1984), SDS-PAGE patterns of solubilized S-layers revealing more than one band should be explained, not ignored. They suggest that low molecular weight species result from proteolytic degradation of surface array proteins during isolation, due in part, to the use of denaturants in many isolation schemes (it is obvious that a degraded protein is of little use for chemical or physical characterization). There is good evidence for such a proteolysis problem with the S-proteins of *A. serpens* VHA and *A. serpens* MW5 (Kist and Murray, 1984; Koval and Murray, 1984) as well as *S. ureae* (Baumeister *et al.*, 1981). The degradation of the S-protein of *A. serpens* VHA appears to be a non-specific proteolytic degradation. If urea extracts of cell walls are examined by SDS-PAGE, without prior removal of the urea, only one band is seen, but if the urea is first removed by dialysis, degradation of the protein is observed. Baumeister *et al.* (1981) reported that when the S-layer protein of *S. ureae* was prepared in the cold, only one protein band was seen by SDS-PAGE but when prepared at 20°C, multiple bands were evident suggesting extensive degradation.

Additional protein bands on SDS gels have been linked to other explanations such as coextraction of other proteins, especially in crude preparations, but this explanation is difficult to accept in more highly purified preparations. For example, Kupcu *et al.* (1984)

extracted the S-protein of *B. stearothermophilus* with 5M guanidine/HCl and further purified it by Sepharose CL-6B chromatography yet SDS-PAGE revealed four prominent bands ranging from 80K to greater than 160K. One explanation which has been put forward for these multiple bands on SDS gels is microheterogeneity in S-protein glycosylation. This explanation necessitates demonstrating that the protein is a glycoprotein and is usually done by periodic acid-Schiff(PAS) staining in SDS gels. However, Thornley *et al.* (1974) found artifacts using this approach, i.e., the protein stained PAS positive in SDS gels but not in native gels. Nevertheless, PAS positive S-proteins have been found for *Campylobacter fetus* (Winter *et al.*, 1978), *C. thermohydrosulfuricum* and *C. thermosaccharolyticum* (Sleytr and Thorne, 1976) and *B. stearothermophilus* (Kupcu *et al.*, 1984) and may explain the origin of multiple bands on SDS-PAGE in some cases.

Only one purification protocol has involved the use of S-protein recovered from culture supernatant (Winter *et al.*, 1978). These workers purified the S-protein of *C. fetus* from culture supernatant using free flow electrophoresis. The final preparation produced more than one band by SDS-PAGE but this was probably due to a microheterogeneity in a carbohydrate moeity (Winter *et al.*, 1978). Purification of S-proteins from culture fluids may eventually replace more labourious protocols which begin with the cell wall fraction and would obviate the need to expose the protein to a denaturing agent which could lead to an increased susceptibility to proteolytic degradation (Koval and Murray, 1984). A number of S-proteins are secreted and can be recovered from culture fluids. Organisms which are known to secrete their S-layer proteins into the culture fluids include *Acinetobacter* 199A (Thorne *et al.*, 1976), *A. serpens* VHA (Koval and Murray, 1985), and *B. sphaericus* P-1 (Howard and Tipper, 1973). Belland and Trust (1985) constructed mutants of *A. salmonicida* by Tn5 mutagenesis which synthesized LPS molecules lacking O-side chains. The S-protein of these mutants was synthesized and exported but could not be assembled on the cell surface; large quantities of S-protein were recovered from the culture fluids in nearly pure form.

1.4 *In vitro* self-assembly of S-layer proteins

Most S-layer proteins have been demonstrated to self-assemble *in vitro* into two-dimensional arrays indistinguishable from those present on the cell surface. The following discussion is divided into S-proteins of gram-negative and gram-positive bacteria because there does appear to be a correlation with *in vitro* reassembly characteristics.

The S-proteins of the gram-positive species *C. thermohydrosulfuricum*, *C. thermosaccharolyticum* (Sleytr and Glauert, 1976), *L. buchneri* (Masuda and Kawata, 1981) and *B. brevis* S1 (Abe *et al.*, 1983) will reassemble in solution, simply upon removal of the guanidine/HCl or urea, by dialysis against distilled water. The S-proteins of *B. sphaericus* 9602 (Hastie and Brinton, 1979a , *L. brevis* (Masuda and Kawata, 1980) and *B. stearothermophilus* (Jaenicke *et al.*, 1985) have been reassembled in solution by dialysis against Tris/HCl or sodium phosphate buffers; it is not known whether distilled water would work just as well.

Unlike the S-proteins of gram-positive bacteria, those of the gram-negative bacteria must be induced to self-assemble after removal of the denaturant. These inducements usually involve a suitable interfacial surface and ionic environment. The S-protein of *Acinetobacter* 199A will reassemble at an air/water interface in the presence of Cl^-; larger polyvalent anions ($S0_4^{2-}$, $P0_4^{3-}$) inhibit self-assembly (Thorne *et al.*, 1975). The double S-layer of *A. serpens* MW5 requires Ca^{2+} for reassembly but it is not clear whether a suitable interface is necessary (Kist and Murray, 1984). Like strain MW5, *A. serpens* VHA reassembles in the presence of Ca^{2+} but requires an interface composed of phospholipid and lipopolysaccharide (Chester and Murray, 1978). The LPS species can be prepared from *A. serpens* VHA or *Pseudomonas aeruginosa* indicating that the interaction is non-specific with the interface probably providing a suitable charge density.

Disregarding the particular aspect of *in vitro* self-assembly for the moment, it is clear that S-proteins fall roughly into two classes: those which are inherently insoluble in aqueous buffer systems and those which are readily soluble, provided the triggering conditions for self-assembly are absent. Unfortunately, the insoluble S-proteins have received more attention than the soluble variety. For example, Hecht *et al.* (1986) in their study of the S-protein from the archaebacterium *Halobacterium halobium* state that "surface layer proteins from eubacteria [which] are intrinsically insoluble in aqueous buffer solutions". In fact, the S-proteins from the gram-negative organisms *A. salmonicida* (Phipps *et al.*, 1983), *Acinetobacter* 199A (Thorne *et al.*, 1975), *A. serpens* VHA and MW5 (Buckmire and Murray, 1973; Kist and Murray, 1984), and *C. fetus* (Winter *et al.*, 1978) are all soluble in certain aqueous buffer systems. Just as the loss of solubility by some S-proteins of gram-negative organisms is triggered by the appropriate ions and interfacial surface, the solubility of the S-protein of *H. halobium* decreases with increasing NaCl concentration. The distinct difference in solubility properties between S-proteins of gram-negative and gram-positive bacteria might be expected to be reflected in the amino acid sequence of the proteins and perhaps in the amino acid composition itself, i.e., some proteins may exhibit a more highly hydrophobic composition thus leading to a lower solubility. There is no primary sequence information available on any S-protein but amino acid composition has been determined for a number of molecules (see Sleytr and Messner, 1983); all possess between 48-55% hydrophobic amino acids. Bhowmik *et al.* (1985) reported that the S-protein of *L. acidophilus* possessed only 31% hydrophobic amino acids. However, only a 24h hydrolysis period was used and quantitative liberation of hydrophobic amino acids requires a hydrolysis period in excess of 24h. All other S-proteins from lactobacilli possess 48-50% hydrophobic amino acids (Masuda and Kawata, 1983).

While reassembly of S-proteins is an interesting property, it has been overemphasized in the literature. Reassembly products have been characterized in great detail using electron microscopy as to their sizes, shapes, and dimensions but these studies would seem to shed little light on the state of the protein on the cell surface. Jaenicke *et al.* (1985) recently extended the electron microscopic analysis of the reassembly products with a kinetic analysis of their formation. It is difficult to see how such studies can contribute to an understanding of the *in vivo* assembly process, which is presumably the objective of studying the *in vitro* reassembly process in the first place. Unlike *in vitro* reassembly which occurs instantaneously from a denatured protein, S-layers *in vivo* never form on the cell surface *de novo*, by instantaneous crystallization of the entire layer using a large pool of available S-protein. *In vivo*, subunits are added continually, as the cell surface area increases with a continual recrystallization of the S-layer.

1.5 Interactions between S-layer proteins

In vitro self-assembly experiments indicate that the major driving force for assembly of the S-layer is the so-called hydrophobic effect (Tanford, 1978). In this respect S-layer organization is no different from the assembly of any other oligomeric protein composed of identical subunits (Cantor and Schimmel, 1980).

A number of studies have attempted to examine the types of bonds which hold S-layers together by evaluating the effect of various perturbants on S-layer structure. This is a very different type of experiment than conducting *in vitro* reassembly experiments because the starting material is the S-layer assembled by the cell. As will be discussed below, S-layers reassembled in the laboratory can exhibit very different stability from those assembled *in vivo*. Most of the experiments evaluating the effects of various disrupting agents begin with the isolated cell wall fraction carrying the attached S-layer. It is obvious from this approach that it is experimentally difficult, or impossible, to separate the two possible effects the disrupting agent is having: (1) disruption of subunit-subunit interactions, (2) subunit-cell wall interactions or both. Only if the S-layer is removed intact from the cell wall can a statement be made that a cell wall-subunit interaction has been affected. Similarly, only if the S-layer is disorganized and at the same time remains attached to the cell wall can a statement be made that subunit-subunit interactions have been affected.

If the perturbant has no effect on either attachment or organization a qualified conclusion may be made that neither interaction is affected. This is because the association of the S-layer with the underlying surface may stabilize it, so even if some bonds are disrupted no disorganization results. Perhaps the most extreme example of this point is the S-layer of *D. radiodurans*. The S-protein of *D. radiodurans* has been shown to be literally cut to pieces *in vivo* yet retains its organized state. When the S-layer of this organism is solubilized with SDS at 100°C and analyzed by SDS-PAGE multiple bands are seen. As hinted above, the clear bias in the literature is that S-layers are formed from single proteins. This situation would be expected from molecular complexes with a high degree of symmetry. There is good evidence that proteolysis occurs *in vivo* but the cleaved polypeptides remain associated with one another in the native S-layer. Upon dissociation with SDS, the cleaved peptides come apart producing multiple bands on SDS-polyacrylamide gels (Rachel *et al.*, 1983).

As mentioned, in order to determine the nature of the interactions between S-layer protein subunits, S-layer sheets must be isolated intact from cell walls. This has only been accomplished for the S-layers of the gram-positive bacteria *S. ureae* (Beveridge, 1979), *B. polymyxa* (Nermut and Murray, 1967), *B. sphaericus* P-1 (Aebi *et al.*, 1973) and two *Clostridium* species (Sleytr and Glauert, 1976). In these cases, intact S-layer sheets were recovered by digesting the peptidoglycan from beneath the S-layer or by treating the cell wall with a low concentration of urea or guanidine/HCl which detaches fragments of the S-layer intact.

In all cases, intact S-layer fragments dislodged from cell walls could be disintegrated by acidification which would disrupt ionic interactions between charged residues of the protein molecule. The *S. ureae* array was not disrupted by dithiothreitol or ß-mercaptoethanol indicating disulfide bonds were not important to the structure. This is not surprising since no cysteine has been found in this S-protein or any S-protein from the so-called typical gram-negative and gram-positive eubacteria (Sleytr and Messner, 1983). Similarly, disulfide bond perturbants have not been found to affect any cell wall associated surface layer so far examined indicating that this is probably a general phenomenon. Beveridge (1979) and Nermut and Murray (1967) examined the effects of a number of detergents differing in their abilities to disrupt hydrophobic interactions. Triton X-100 and Sarkosyl were not effective while not surprisingly SDS disrupted the arrays. Triton X-100 has not been found to have any effect on any cell wall associated S-layer probably because it does not readily disrupt protein:protein interactions (Helenius and Simons, 1975). The effects of urea and guanidine/HCl were tested on all the S-layers listed above and found to be potent disrupting agents. Although it is stated repeatedly in the S-layer literature that urea and guanidine/HCl disrupt hydrogen bonds, this is not correct. These agents act by increasing the solubility of hydrophobic side chains in the solvent and thus primarily disrupt hydrophobic interactions (Cantor and Schimmel, 1980).

One eubacterial S-layer that stands out in its resistance to chemical perturbants is that from *D. radiodurans* strain Sark (Thompson *et al.*, 1982) and strain R1 (Baumeister *et al.*, 1982). The exceptional stability of the S-layer of this organism allows other envelope components to be solubilized with 2% SDS at 20°C for 12h (Thompson *et al.*, 1982). Alternatively, S-layer sheets can be released from whole cells by treatment with SDS (1%) at 60°C for 2h and purified by isopycnic density gradient centrifugation (Baumeister *et al.*, 1982). The resulting array can be disrupted by acidification and dissociated by urea, guanidine/HCl and SDS but only at temperatures approaching 100°C (Baumeister *et al.*, 1982).

With the exception of *D. radiodurans* and the lack of disulfide bonding, none of the data described above is particularly striking. It is not surprising that dissociation of the protein array results from disruption of hydrophobic and ionic interactions.

1.6 Interactions between S-layer proteins and other cell wall components

1.6.1 Gram-positive cell walls

A number of so-called heterologous reattachment experiments have been performed with S-layer proteins from gram-positive organisms. These experiments involve determining whether an S-protein from one organism can reassemble on the naked cell wall of another. This 'Frankenstein' type of experiment should give an indication whether a global feature of gram-positive cell walls is responsible for S-layer association. Heterologous reattachment (and reassembly) has been shown for two *Clostridium* species (Sleytr, 1975; Sleytr, 1976) and a few *Lactobacillus* species (Masuda and Kawata, 1980;

Masuda and Kawata, 1981). However, not all *Lactobacillus* cell walls supported reattachment of a heterologous S-layer derived from another *Lactobacillus* species, indicating species or strain differences were important. Hastie and Brinton (1979b) are the only workers who have tested for heterologous reattachment between different genera. They found that the S-protein from *B. sphaericus* 9602 would only reattach and reassemble on its own naked cell wall and not that of other *Bacillus*, *Corynebacterium*, *Lactobacillus* or *Staphylococcus* species (or strains).

Masuda and Kawata (1981, 1985) attempted to identify components of the *L. buchneri* cell wall responsible for S-layer attachment. The S-protein of this organism was found to reassemble on cell walls treated with cold trichloroacetic acid which removed teichoic acid, but not on walls extracted with hot formamide to remove both a neutral carbohydrate component and teichoic acid, leaving only the peptidoglycan. They concluded that the S-layer was bound to the neutral carbohydrate moiety.

The few studies outlined above indicate that interactions responsible for attachment of S-layers to the rest of the gram-positive cell wall are obscure. Recent experiments by Masuda and Kawata (1985) illustrate the problem in determining meaningful information in this area. These workers modified the S-protein of *L. buchneri* by amidination, acetylation, succinylation and amidation. Only the amidinated protein retained the ability to reassemble on cell walls. They concluded, not surprisingly, that both the positive charge on the amino groups and the negative charge on the carboxyl groups were important. This type of approach lacks precision because except for the amidinated protein, all the other modifications caused the protein to lose the ability to self-assemble in the absence of a cell wall template indicating major modifications to the protein had occurred.

One point is clear, the bonds formed between the rest of the cell wall are different from those formed between adjacent subunits. This is because acidification of a cell wall suspension produces S-layer disorganization but leaves the protein attached to the cell wall surface (Hastie and Brinton, 1979a, 1979b; Sleytr and Glauert, 1976). While their assembly *in vitro* is no doubt driven by hydrophobic interactions, association with the cell wall provides even more stability to the array since *in vitro* self-assembled products (not attached to the cell wall) are fragile and disintegrate easily (Sleytr, 1976). Similarly, Hastie and Brinton (1979b) found that it took higher concentrations of urea to dissociate cell wall associated arrays of *B. sphaericus* 9602 than the non-anchored variety.

1.6.2 Gram-negative cell walls

Generally, the approach taken with gram-positive cell walls has been applied to gram-negative cell walls. There are only a few studies in this area and the most complete is that of Thorne *et al.* (1975) who studied the S-layer of *Acinetobacter* 199A. Cell walls extracted with EDTA to remove LPS, or digested with phospholipase C to hydrolyze phosphatidylethanolamine and phosphatidylglycerol, still supported S-protein reattachment and reassembly. Similarly, the S-protein could not be induced to reassemble when presented with purified LPS. These experiments indicated neither phospholipid nor LPS were the sites of S-protein attachment and a protein component was assumed to be the receptor. Chester and Murray (1978) had demonstrated earlier that the S-layer of *A. serpens* VHA could be induced to crystallize on vesicles formed of phospholipids and LPS from this organism, again suggesting a role for LPS in S-layer attachment. Recently, Belland and Trust (1985) showed that mutants of *A. salmonicida* unable to produce smooth type LPS were also unable to assemble their surface layer. The S-protein was exported, but failed to bind to the cell surface and was found in the culture fluids.

A major difference between the S-layers of gram-positive and gram-negative bacteria is their response to the chelating agents EDTA and EGTA. Neither of these chelating agents has been found to have any effect on native S-layers of gram-positive organisms. There may be two exceptions which will be discussed later in this thesis. In contrast, the S-layers of *A. serpens* VHA (Koval and Murray, 1983), *Acinetobacter* 199A (Thorne *et al.*, 1971) and two marine ammonia oxidizing bacteria *Nitrosomonas* and *Nitrosocyctis* (Watson and Remsen, 1969) are disrupted by these agents which suggests a role for divalent cations in S-layer structure. Divalent cations may form a salt bridge between suitably disposed carboxyl groups within the S-protein molecule itself, between S-protein molecules or between the S-protein and the outer membrane (Buckmire and Murray, 1976). Thorne *et al.* (1975) tried to confirm this salt bridge idea by blocking and modifying carboxyl groups located in both the cell wall and S-protein of *Acinetobacter* 199A but the results obtained were not conclusive.

A definite requirement for divalent cations, specifically Ca^{2+} has been shown for the genus *Aquaspirillum* (Beveridge, 1981). Both *A. serpens* VHA (Koval and Murray, 1985) and *A. putridiconchylium* (Beveridge and Murray, 1976) possess no visible S-layer when grown in a medium lacking Ca^{2+}. Unlike the aquaspirilla, the involvement of particular divalent cations in the S-layers of other gram-negative bacteria has not been established.

1.7 S-layers and prokaryotic diversity

Although S-layers are a surface structure one should not regard their overall structural similarities superficially. Simply because two proteins pack in a geometrically regular pattern at a gross level does not necessarily indicate a similarity of fine structure or function. For example, the hexagonally (or tetragonally) packed photosynthetic reaction complexes of the internal membranes of *Rhodopseudomonas viridis* appear almost indistinguishable on a gross level from typical bacterial surface layers (Miller, 1979; Welte *et al.*, 1981). In other words, appearances can be deceiving. While this statement may seem obvious, microbiologists in particular have been guilty of ignoring it. The heavy emphasis placed on morphology as a basis for bacterial taxonomy has been shown to be misleading (Stackebrandt and Woese, 1981) resulting in the misclassification of many species. Similarly, a variety of cell wall structures have been classed as S-layers, based solely on their 'regularity'; some of these structures have quite different properties from the S-layers already described. The preceding discussion has been deliberately restricted to the traditionally defined gram-positive and gram-negative eubacteria whose S-layers conform to a definition first proposed implicitly by Chalcroft *et al.* (1986). That is, an S-layer is a superficial regular protein (or glycoprotein) array interconnected by noncovalent forces and whose integrity does not depend on interactions with the underlying cell wall.

Structures classed as S-layers have been described in most of the ten major eubacterial lines of descent determined by 16S rRNA cataloguing (Sleytr and Messner, 1983; Woese *et al.*, 1985). One line of descent is represented by *D. radiodurans*. While the hexagonal S-layer of this organism appears grossly similar to other eubacterial S-layers it is quite a different structure as indicated by its exceptional resistance to solubilization by many denaturants (Baumeister *et al.*, 1982; Lancy and Murray, 1978). Rather than considering this S-layer to be composed of monomers grouped into a hexameric building block, Baumeister and co-workers (Baumeister *et al.*, 1986; Rasch *et al.*, 1984) consider the S-layer to be built from dimers with one monomer from one hexamer and one monomer from another. However, in this case the delineation may be quite arbitrary. The array is so refractory, that a dissection of its substructure is not possible without use of treatments which completely dissociate it irreversibly into monomers. The S-layer of *Chlamydia trachomatis* has been examined using image processing techniques and is of the M_2C_3 type (see Baumeister *et al.*, 1986). This is clearly different from all other hexagonal

S-layers so far described (Fig. 1-6). Hexagonal S-layers of the M_6C_3 or M_6C_2 type are widely distributed taxonomically. They are found in *Deinococcus*, gram-positive eubacteria, gram-negative eubacteria and even in the cyanobacteria and are therefore popular designs. The S-layer of *C. trachomatis* is believed to possess as its major component the so-called major protein of the outer membrane (Chang *et al.,* 1982). This protein has been shown to be present as oligomers, cross-linked via disulfide bonds (Newhall and Jones, 1983). This fact makes this S-layer unique in another respect, the protein molecules of the layer are covalently associated with one another. Unlike all other bacteria listed so far, *C. trachomatis* does not possess peptidoglycan yet is osmotically stable. This is believed due to extensive disulfide cross-linking of outer membrane proteins which presumably serves the same function as peptidoglycan, providing rigidity to the cell wall (Hackstadt *et al.*, 1985). However, the concensus for all other eubacteria is that S-layers serve no morphogenic function (Sleytr and Messner, 1983) since mutants lacking their S-layers are apparently healthy. Thus, the unique organization of the S-layer of *C. trachomatis* may be related to a specific morphogenic function not required by other eubacteria. The spirochetes are another major line of eubacterial descent reported to possess S-layers (Sleytr and Messner, 1983). However, the regularly arranged protein subunits of the spirochete cell wall may be actually located within the bilayer of the outer sheath and may be more analogous to the *E. coli* matrix protein (Masuda and Kawata, 1982) than an S-layer *per se* .

Even within the traditionally defined gram-negative eubacteria S-layer-like structures can be found. The so-called S-layers of the pseudomonads are somewhat confusing. Baumeister *et al.* (1986) classified the surface array of *P. avenae* (Wells *et al.*, 1983) as an M_4C_4 type which means that protein mass should be centered around both of the four-fold rotational axes. However, in a subsequent study (Chalcroft *et al.,* 1986) of the *P. acidovorans* S-layer which has the same structure in projection as the *P. avenae* layer, both four-fold axes clearly fall in pore regions (Fig. 1-7). In fact, it is very difficult to see how either array could be of the M_4C_4 type since there is no obvious connectivity between the morphological subunits at all. For this reason Chalcroft *et al.* (1986) further point out that the S-layer of *P. acidovorans* (and by implication that of *P. avenae*), may not be true S-layers, since all true S-layers possess interconnected subunits. Thus, the "S-layers" of these two *Pseudomonas* species may be more correctly termed "regularly arranged outer membrane proteins".

Although the phylogenetic position of *C. trachomatis* is now being defined (Weisburg

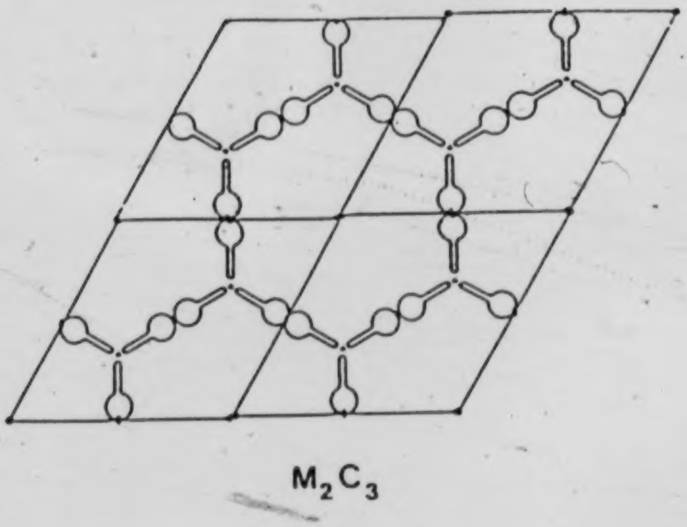

Fig.1-6. The organization of the hexagonal S-layer of *C. trachomatis* .

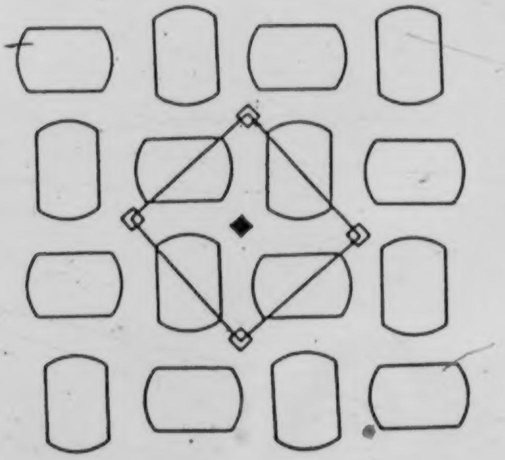

Fig.1-7. Organization of the tetragonal S-layers of *P. avenae* and *P. acidovorans* . Note the "cobblestone" arrangement of the dimeric subunits and their lack of interconnectivity.

et al., 1986), it is interesting that many of the prokaryotic lineages discussed above possesses a distinctive cell wall structure (Holt, 1978; Sleytr and Glauert, 1982) and cytoplasmic membrane lipid composition (Goldfine, 1982). These differences in cell envelope structure and composition seem to include S-layer characteristics as well and rather than looking at the S-layer as a separate entity, it should be viewed in the context of the entire cell envelope.

1.8 Thesis objectives

Azotobacter vinelandii, a gram-negative bacterium commonly isolated from soil, has been reported to possess an S-layer of tetragonal organization (C.S. Fuqua, K.S. Howard, and M.D. Sokolofsky, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, J21, p.92). Few tetragonal surface layers from gram-negative bacteria have been characterized from both a structural and biochemical standpoint. The only S-layers of this type which have been, or are now being studied, are those from *Acinetobacter* 199A (Thornley, 1975) and *A. salmonicida.* The S-layer of the former organism is no longer being actively studied although the latter structure is under intensive investigation. The surface array of *A. vinelandii* provides a good opportunity to study a tetragonal S-layer of a gram-negative organism. While little is known about the cell wall of *A. vinelandii* beyond the fact it is a "typical" gram-negative type (Sadoff, 1975), much preliminary data exists on both biochemical (S.P. Schenk 1978; Ph.D. Thesis , University of Texas at Austin, TX) and structural (J.L. Doran 1983; Ph.D. Thesis, University of Alberta, Edmonton, AB) aspects of its surface array. A detailed understanding of its structure should aid in formulating general principles concerning the organization and structure of bacterial surface layers.

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2. Old Bottles, New Wine

2.1 Introduction

In 1977, Schenk *et al.* described a protein of 60,000 apparent molecular weight (designated "60K") which copurified with the envelope fraction (inner and outer membrane) prepared from *Azotobacter vinelandii* 12837. Analysis of total envelope protein by SDS-PAGE and densitometry indicated 60K accounted for 20% of the total envelope protein. Further, immunoelectron microscopy showed the protein was exposed on the outer surface of the outer membrane. The association of 60K with the outer membrane was found to be easily disrupted because the protein could be extracted from whole cells by washing with distilled water; this indicated 60K was probably a peripheral outer membrane protein. Preliminary experiments also suggested that divalent cations effected the intermolecular interactions of 60K, possibly through its attachment to the outer membrane (Schenk and Earhart, 1981). The amino acid composition of 60K was determined and found to bear similarities to the *Acinetobacter* 199A S-layer protein (Thornley *et al.*, 1974). These data lead Schenk and Earhart (1981) to propose that 60K formed a regular surface layer on *A. vinelandii* 12837. This proposal became accepted in the literature (van Iterson, 1984; Lugtenberg and Van Alphen, 1983) although no attempt was made to visualize the layer using electron microscopy, in order to confirm it had a 'regular' structure. Such conclusions are hazardous since all proteins with the properties listed above do not form regular arrays (Hayes, 1984; Inouye *et al.*, 1979, 1981; Takumi *et al.*, 1983; Word *et al.*, 1983) although they may form surface layers. Interestingly, *Azotobacter vinelandii* 12837 has been shown to possess a regular surface layer of tetragonal organization (C.S. Fuqua, K.S. Howard, and M.D. Socolofsky, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, J21, p. 92.); apparently this report has not been widely noted.

Page and Doran (1981) found that 60K recovered from distilled water wash fluids was an acidic protein (pI 5.1) which could be precipitated by Ca^{2+} or Sr^{2+} but not by Mg^{2+}. The precipitation of 60K by divalent cations is similar to results obtained with the S-protein of *Acinetobacter* 199A (Thorne *et al.*, 1975) but the specificity of the reaction, i.e., with Ca^{2+} and not Mg^{2+} is interesting, and suggests a specific interaction between

A version of this chapter has been published. Bingle, W.H., J.L. Doran and W.J.Page. 1984. Regular surface layer of *Azotobacter vinelandii*. J. Bacteriol. 159: 251-259.

60K and Ca^{2+}. The significance of this finding is reinforced by the observation that *A. vinelandii* OP grown in a medium lacking Ca^{2+}, possesses no tetragonal surface layer when viewed by freeze-etch electron microscopy, but when Ca^{2+} (or Sr^{2+}) but not Mg^{2+} is added to the culture fluids, the layer rapidly appears completely covering the cell surface (W.H. Bingle, J.L. Doran, and W.J. Page. Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, K155, p. 202). A similar type of specific interaction with Ca^{2+} has also been noted for the surface array protein of the *Aquaspirillum putridiconchylium* (Beveridge and Murray, 1976). The ability of the 60K protein to specifically complex Ca^{2+} appears to be a reasonable candidate for a measure of the 'activity' of the protein, i.e., an indicator of whether the protein is present in its native state and is biologically active.

To date, no direct evidence has been presented for the involvement of the 60K protein in a regular surface layer reported present on *A. vinelandii*.; this chapter provides such evidence. This chapter also explores: (1) conditions for optimizing the release of the 60K protein from whole cells by distilled water washing, (2) the use of Ca^{2+}-precipitation as an indicator of biological activity (*in vitro* reassembly competence) of the 60K protein and (3) an evaluation of the state of outer membrane after distilled water washing. It is important to appreciate the condition of the outer membrane, after removal of 60K, should a template be required for the study of *in vitro* reassembly of the *A. vinelandii* surface layer protein. ~~Finally, (4)~~ attempts at establishing conditions for the *in vitro* reassembly of the S-layer are described.

2.2 Materials and Methods

Bacterial strains and growth conditions

In this study the capsule-negative strain UW1 (Nif^-) of *A. vinelandii* OP (ATCC 13705) described by Fisher and Brill (1969) was used. Liquid cultures (40% culture volume per flask volume) of modified Burk medium (Page and Doran, 1981) were grown at 30°C in a gyratory water bath shaker (model G-76; New Brunswick Scientific Co. Inc., Edison, N.J.) operating at 300 rpm. Modified Burk medium contained 1% glucose, ammonium acetate (1.1 g/l) as a nitrogen source and $CaCl_2$ in place of $CaSO_4$. Burk buffer (pH 7.2) was Burk medium without added glucose or ammonium acetate and contained 4.6mM K_2HPO_4, 1.6mM KH_2PO_4, 0.8mM $MgSO_4$, 0.6mM $CaCl_2$ and 18μM $FeSO_4$. OFeBB was Burk buffer lacking iron and $BBPO_4$ was Burk buffer potassium phosphate.

Liquid cultures were inoculated to an initial optical density at 620 nm of 0.05 and were grown for 20-24h to a final optical density of approximately 3; optical density measurements were made with a Bausch and Lomb Spectronic 20 spectrophotometer. Iron-limited cells were grown as described by Page and von Tigerstrom (1982).

Outer membrane isolation

Cells were broken using a French pressure cell and fragments of outer membrane were separated from inner membrane and poly-ß-hydroxybutyrate (PHB) granules on sucrose gradients according to the method of Page and von Tigerstrom (1982).

Reattachment of S-protein to outer membrane vesicles (OMV)

Outer membranes were recovered from sucrose gradients (Page and von Tigerstrom, 1982) and washed by centrifugation with 8 mM Tris/HCl, pH 7.8 to remove the sucrose. Membrane material (1.5 mg membrane protein) was suspended in 1.5 ml of distilled water at room temperature and vortexed vigorously for 1 min. Chloride salts of Na^+, K^+, Ca^{2+}, Mg^{2+}, Sr^{2+}, and the nitrate salt of Be^{2+} were then added, and after 2 h of incubation at room temperature, the outer membrane fragments were recovered by centrifugation at 100,000 g for 30 min. Na^+ and K^+ were added to a concentration of 10, 5, 2, 1, 0.5, 0.2, 0.1, or 0.05 mM while Be^{2+}, Mg^{2+}, Ca^{2+}, and Sr^{2+} were added to a concentration of 3.4, 1.7, 0.68, 0.34, 0.17, 0.068, 0.034, or 0.017 mM. Reattachment of protein to the outer membrane was monitored by SDS-PAGE and by measuring the reduction in supernatant protein concentration.

Washing intact cells with distilled water

Cells were harvested from culture fluids by centrifugation in a Sorvall RC-5 centrifuge at 15,000 g for 5 min at 4°C. The cells were suspended in 1 volume of ice-cold OFeBB and pelleted again. Each cell pellet was resuspended to and optical density (OD) at 620 nm of 3 in either ice-cold distilled water (Protocol 1) or fresh OFeBB (Protocol 2). Ten-millilitre portions were collected by filtration on a 47 mm diameter Millipore filter (0.45 µm (Protocol 1) or 0.8 µm (Protocol 2) pore diameter) and the cells were washed either 4 (Protocol 1) or 5 (Protocol 2) times with 10 ml of distilled water preheated to 38°C, 44°C or 46°C. The filter was floated on ice-cold OFeBB to lift the cells from the filter and the cells were collected by centrifugation at 15,000 g for 15 min at 4°C.

Concentration and desalting of distilled water wash fluids

Distilled water wash fluids were filtered through a 0.45 µm Millipore filter and concentrated by lyophilization. The resultant white flocculant material was resuspended to a protein concentration of 1 mg/ml in distilled water. This preparation was desalted either by dialyzing (10,000-12,000 molecular weight cut-off) for 24 h against three changes of 1000 volumes of distilled water at 4°C or by gel filtration using Sephadex G-25 (Pharmacia Fine Chemicals, Upsala, Sweden). In some cases desalting was accomplished using the Amicon ultrafiltration-micropartition system (YMT membrane; size cut-off 10,000; Amicon Corp., Oakville, Ontario).

Freeze-etch electron microscopy

Cells used for freeze-etch electron microscopy were pelleted and resuspended in the residual supernatant fluid following decantation. A drop of thick cell suspension on a 3 mm diameter gold disk was frozen in liquid Freon-22 and stored in liquid nitrogen (Moor, 1969). The specimens were held at -100°C and were fractured and etched for 60 sec (DeVoe *et al.*, 1971) , using a Balzers BA 360M apparatus. Replicas were formed by shadowing with platinum (200-250 nm) and carbon (2 -2.5 µm). The organic material contaminating the replicas was removed by treatment with concentrated sulfuric acid, followed by distilled water, commercial bleach and a final rinse with distilled water. The replicas were mounted on 200-mesh copper grids and observed with a Philips 300 electron microscope at an accelerating voltage of 60 or 80 kV.

Radioiodination of whole cells

Cells were resuspended at a concentration of 1 µg of total cell protein per µl in OFeBB and incubated with shaking (100 rpm) for 30 min at 30°C before labelling. A number of whole cells equivalent to 50 µg of cell protein was mixed with ^{125}I and enough OFeBB to bring the volume to 100 µl. This suspension was mixed and added to the bottom of a tube (10 X 75 mm) onto which Iodo-Gen (1, 3, 4, 6-tetrachloro-3α,6α-diphenylglycouril, Pierce Chemical Co., Rockford Ill.) had been coated by drying down a solution (1 or 2 mg/ml) in methylene chloride at 40°C (Fraker and Speck, 1978).

Radioiodination continued for 10 min at 30°C. The cell suspension was then transferred to a 1.5 ml Eppendorf centrifuge tube and the cells were centrifuged in a Fisher 235 microfuge for 3 min. The resulting cell pellet was washed three times by centrifugation with OFeBB containing 25 mM NaI. The washed cell pellets were resuspended in 150 µl of SDS-sample buffer and analyzed by SDS-PAGE. Autoradiographs were made from wet gels with Kodak XOMAT-AR5 film.

Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted according to the method of Laemmli (1970). Samples were boiled for 7 min in sample buffer containing 2% SDS, 10% sucrose, and 5% ß-mercaptoethanol in 62.5 mM Tris/HCl, pH 6.8 before application to the gel. Slab gels (1.5 mm thick) were formed in a Hoeffer model SE 600 electrophoresis cell. Gels were run at 10 mA for 2.5 h through the 5% stacking gel and at 20 mA for 4 h through the 10% separation gel. Gels were stained for carbohydrate and protein according to Fairbanks *et al.* (1971). Horse radish peroxidase type II was used as a glycoprotein standard. Two-dimensional gel electrophoresis was conducted according to O'Farrell (1975) with the modifications introduced by Bishop *et al.* (1980). The proteins used as molecular weight standards were phosphorylase A (94K), bovine serum albumin (68K), gamma globulin H-chain (50K), ovalbumin (45K), gamma globulin L-chain (23.5K) and ribonuclease (13.7K). All protein standards were purchased from Sigma Chemical Co., St. Louis Mo.

Assays

Protein was determined routinely by the modified Lowry method of Markwell *et al.* (1978) using bovine serum albumin (BSA) as a standard. For whole cells, a predigestion in 1 N NaOH at 90°C for 10 min was conducted according to Hanson and Phillips (1981). Carbohydrate was measured either by the phenol-sulfuric method or the anthrone method (using glucose as a standard) as described by Herbert *et al.* (1971). The concentration of 2-keto-3-deoxyoctanoic acid (KDO) was determined by the method of Keleti and Lederer (1974).

2.3 Results

Visualization of the *A. vinelandii* tetragonal S-layer

Cells washed once with Burk buffer at 42°C (Doran, 1983) failed to exhibit a tetragonal S-layer. After investigating a number of washing protocols, the following procedure was adopted: Cells were resuspended to an OD_{620} of 1 in OFeBB and incubated at 42°C for 5 min. The cells were recovered by centrifugation at room temperature and the wash treatment was repeated an additional four times. This more rigorous washing regime effectively exposed the S-layer to visualization by freeze-etch electron microscopy (Fig. 2-1) and was used in all further experiments for visualization of S-layers assembled *in vivo*. Careful measurements revealed the center to center spacing between the subunits was 12.5 nm rather than the approximately 10 nm reported by Doran (1983).

Figure 2-1 reveals the general features of most freeze-etch replicas. Where the shadowing angle was optimal, areas of the regular array were evident. However, on the windward side of the cell, accumulations of platinum obscured the array while on the leeward side, an absence of platinum rendered the array invisible. The array possessed limited topographical relief and unless the shadowing angle was appropriate it could be missed altogether. Thus, only those replicas which exhibited both under- and over accumulations of platinum were used when evaluating the effects of various treatments on the surface features of *A. vinelandii*.

Effect of distilled water washing on the outer membrane protein composition of *A. vinelandii*

Because Schenk and Earhart (1981) provided insufficient detail in the description of their method for the 38°C-distilled water extraction of 60K from *A. vinelandii* whole cells, two slightly different washing protocols were investigated in the present study (see Materials and Methods). After distilled water washing, the cells were floated from the filter on ice-cold OFeBB, processed for outer membrane isolation and the distilled water wash fluids were concentrated by lyophilization. Examination of outer membrane proteins after distilled water washing protocol #1 (38°C) showed a slight reduction in the amount of a protein of 55,000 apparent molecular weight (Fig. 2-2; lanes 1 and 2). During pipetting of the distilled water and resuspension of the cells on the filter, the water cooled to 32-33°C. Thus, the cells were never really exposed to a temperature of 38°C for more than brief

Fig.2-1. Freeze-etch replica of the convex surface of *A. vinelandii* UW1 showing the regular S-layer (S). OMF, outer membrane fracture face; ◀ , direction of platinum shadowing; Bar, 0.5 μm.

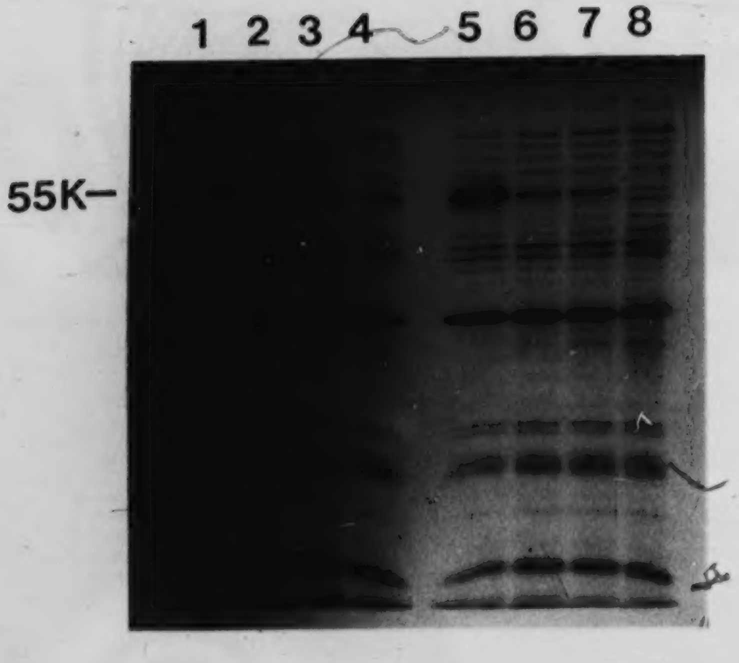

Fig.2-2. Effect of distilled water washing of whole cells on the protein composition of the outer membrane of *A. vinelandii* UW1. *A. vinelandii* whole cells were washed with distilled water either by protocol #1(lanes 1-4) or protocol #2 (lanes 5-8) based on the method of Schenk and Earhart (1981). Cells were processed directly for outer membrane isolation (lanes 1 and 5) or washed with distilled water preincubated at 38°C (lanes 2 and 6), 44°C (lanes 3 and 7) or 46°C (lanes 4 and 8).

period. Therefore, the experiment was repeated with distilled water held at 44°C. During manipulations, the distilled water cooled to approximately 38°C, i.e., when the temperature of the cell suspension over the filter was measured immediately after resuspension of the cells, the temperature was routinely 38°C. This modification to distilled water washing protocol #1 caused almost complete release of the 55K protein from the outer membrane (Fig. 2-2; lane 3). Increasing the temperature another 2°C to 46°C did not appear to aid in the removal of the minor residual 55K protein (Fig. 2-2; lane 4). Total protein recovered from the wash fluids paralleled the impressions gained from following the outer membrane polypeptide composition (Table 2-1).

Surprisingly, distilled water washing protocol #2 produced a completely different pattern of 55K protein release with respect to temperature. Distilled water at 38°C caused almost a complete release of the protein and increasing the temperature to 46°C appeared to allow complete total of the 55K protein from the outer membrane (Fig. 2-2; lanes 5-8). Again, the amount of protein recovered in the distilled water wash fluids corresponded with the reduction in the 55K protein band seen by SDS-PAGE (Table 2-1). It was decided to adopt the modified protocol #2 of Schenk and Earhart (1981) using distilled water preheated to a temperature of 46°C for further work.

When the protein from the distilled water wash fluids was examined by SDS-PAGE, a major 55K protein was found, as well as trace amounts of 45, 30 and 16K polypeptides (Fig. 2-3; lanes 1, 2 and 5). Examination of distilled water washed cells by freeze-etch electron microscopy (Fig. 2-4) showed no evidence of the regular array indicating it was lost concomitant with the removal of the 55, 45, 30, and 16K proteins. Since the 55K protein was the major component of the S-layer it was designated the "S-protein" for future reference. The minor 45, 30 and 16K species were also evident in S-protein preparations produced by a single distilled water extraction of the outer membrane (Fig. 2-3; lanes 3,4 and 6). These proteins also appeared to be released into the culture fluids (Fig. 2-3; lane 7).

Although analysis of outer membranes by SDS-PAGE showed that S-protein appeared to be completely removed from whole cells by this distilled water washing, two-dimensional gel electrophoresis of cell lysates prepared before and after distilled water washing (Fig. 2-5) showed that some S-protein remained with the cell. Whether this residual protein was surface localized, or not yet exported, was unclear.

Table 2-1. Effect of wash temperature on the recovery of protein from *A. vinelandii* UW1 by two distilled water washing protocols based on the method of Schenk and Earhart (1981)[1].

Temperature (°C)	Protein recovered (µg/OD_{620} unit)	
	Protocol #1	Protocol #2
38	6.3	14.0
44	12.6	13.5
46	12.4	15.1

[1] Results of a typical experiment

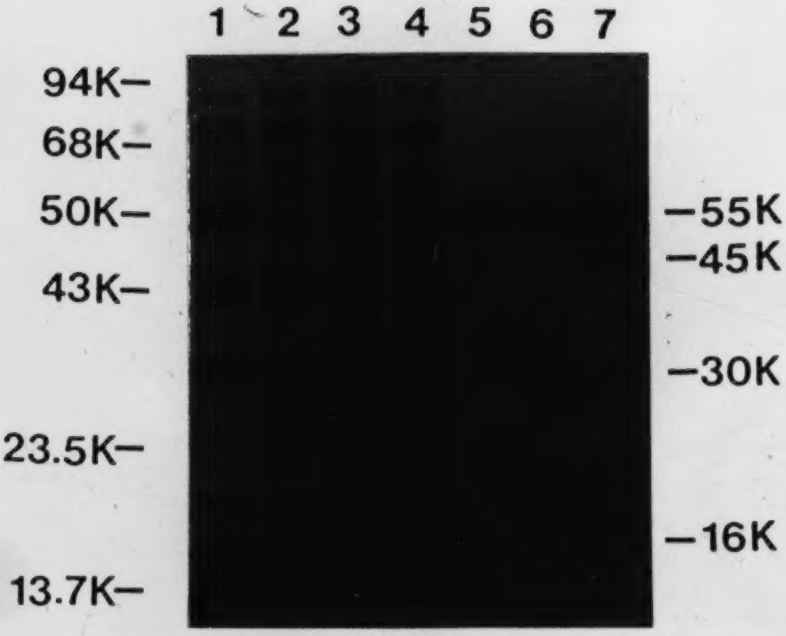

Fig.2-3. SDS-PAGE analysis of outer membrane proteins extracted from *A. vinelandii* UW1 whole cells by distilled water washing. Lanes: 1, outer membranes from unwashed cells; 2, outer membranes from distilled water washed cells; 3, unwashed outer membranes; 4, distilled water washed outer membranes 5, concentrated distilled water wash fluids from whole cells; 6, distilled water extract of outer membrane; 7, concentrated culture supernatant protein.

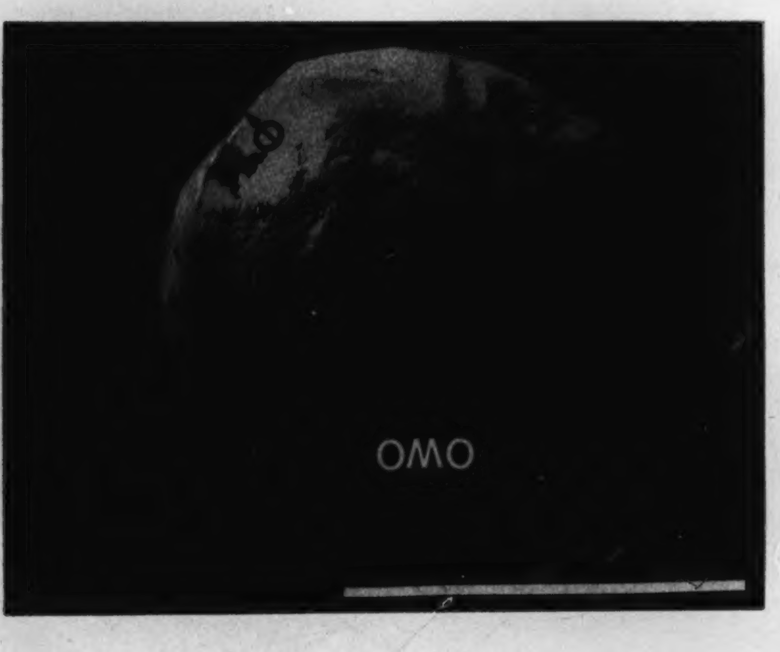

Fig. 2-4. Effect of distilled water washing on the surface features of *A. vinelandii* UW1. Freeze-etch replica, convex surface. OMO, outer surface of the outer membrane; ➘⊖ direction of platinum shadowing; Bar, 0.5 μm.

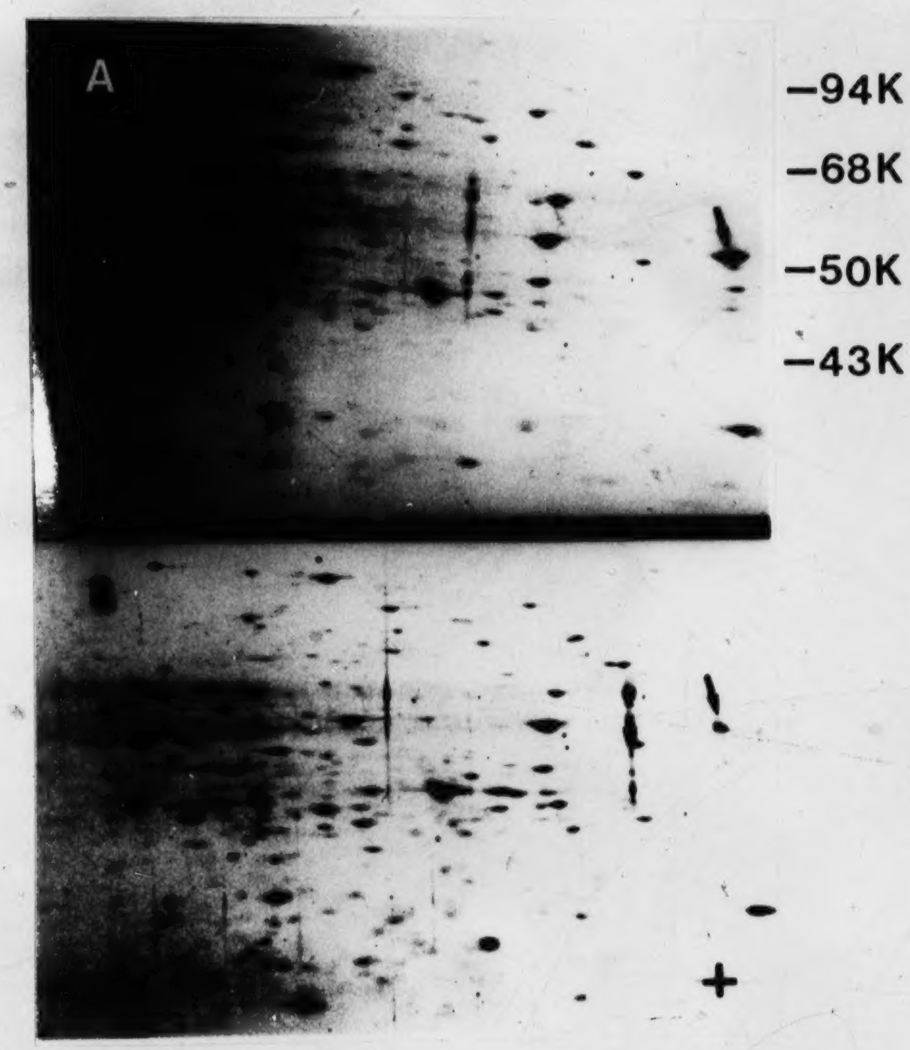

Fig.2-5. Two-dimensional gel electrophoresis of whole cell extracts prepared from unwashed (A) and distilled water washed (B) whole cells. The arrow (↘) indicates the location of an acidic protein of 55, 000 molecular weight that is reduced in abundance when cells are prewashed with distilled water.

Radioiodination of the cell surface

The examination of outer membrane proteins by SDS-PAGE and by two-dimensional gel electrophoresis, after distilled water washing of whole cells, provided somewhat conflicting information on the effectiveness of this treatment for removing S-protein from the cell. The differences could have been due to the fact that the residual S-protein seen on the 2D gels was not yet completely exported. Because distilled water washed whole cells were to be used as a template to study *in vitro* self-assembly, it was important to determine the amount of residual S-protein left on the cell surface. In an effort to confirm that the S-protein was completely removed from the cell surface by distilled water washing, whole cells were radioidodinated to reveal surface exposed polypeptides. Cells radioiodinated with Iodo-Gen (50 µg) and ^{125}I (0.5 µCi/µl) showed a radiolabelled polypeptide of 55,000 molecular weight, but it was not as prominent as expected from the abundance of the S-protein in the outer membrane (Fig. 2-6; lane 1). Since no attempt was made to isolate outer membranes from radioiodinated whole cells, the assignment of any labelled polypeptide to a major outer membrane protein could not be made with complete certainty (Loeb and Smith, 1983). Iron-limited *A. vinelandii* derepress the synthesis of three iron-repressible outer membrane proteins of apparent molecular weight 93,000, 85,000 and 81,000 (Page and von Tigerstrom, 1982) which are probably siderophore receptors. These proteins would be expected to be exposed to the environment and could also serve as markers indicating labelling of outer membrane proteins. As expected, it was possible to label these proteins (Fig. 2-6; lane 2). The failure of S-protein to be detected was not due to its loss from the cell surface during the 25 mM NaI wash since outer membranes isolated from cells so treated, carried S-protein. A major protein of 55,000 molecular weight could be labelled by increasing the amount of Iodo-Gen to 200 µg and the ^{125}I concentration to 5 µCi/µl (Fig. 2-7; lane 1). This polypeptide could be reduced by washing the cells with distilled water before labelling, confirming its identity as the S-protein (Fig. 2-7; lane 2). The proteins apparently labelled under these conditions were few in contrast to labelling under low reagent concentrations. Although it appeared that only the 85K iron-repressible outer membrane protein was labelled under these conditions, this was not the case. The exposure time for the autoradiograph in Figure 2-7 was 15 min as opposed to the 11.5 h needed for the autoradiograph depicted in Figure 2-6. This exposure time was insufficient for detection of labelled integral outer membrane proteins but careful examination of Figure 2-7 showed many faint outer membrane protein bands.

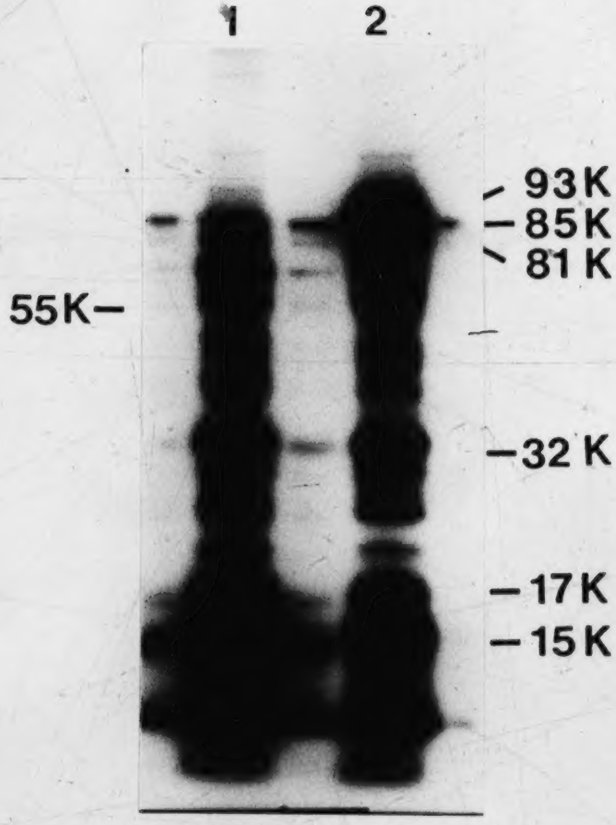

Fig.2-6. SDS-PAGE of radioiodinated whole cells of *A. vinelandii* UW1. Cells were either grown under iron-sufficient (lane 1) or iron-limited (lane 2) conditions and radioidodinated with 50 μg of Iodo-Gen and ^{125}I at 0.5 μCi/μl. The radioautograph was exposed for 11.5 h.

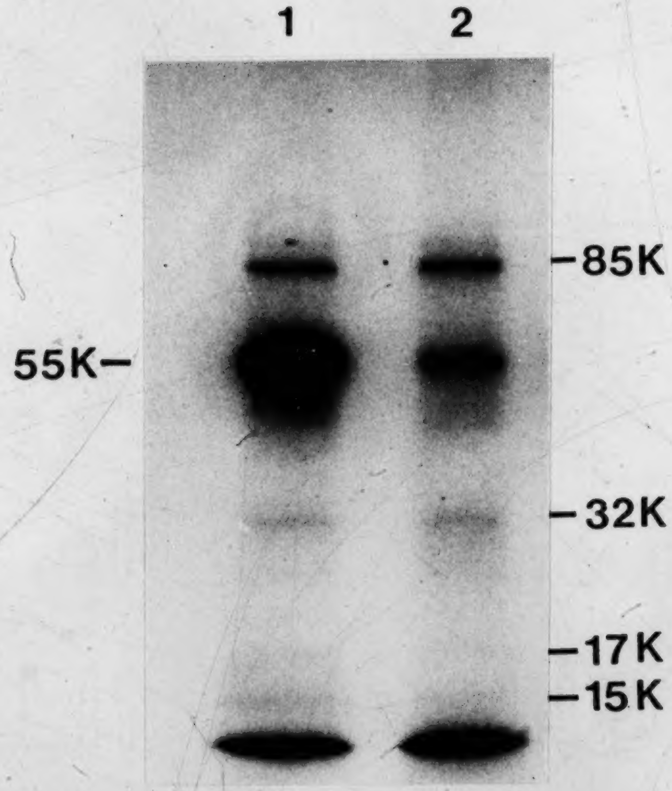

Fig.2-7. SDS-PAGE of radioiodinated *A. vinelandii* UW1 before (lane 1) and after (lane 2) distilled water washing. Cells were grown under iron-limitation and radioiodinated with 200 µg Iodo-Gen and ^{125}I at 5 µCi/ µl. The radioautograph was exposed for 15 min.

Effect of distilled water washing on the outer membrane of *A. vinelandii*

Lipopolysaccharide (LPS) release was used as an indicator of structural damage to the outer membrane caused by the distilled water washing. S-protein concentrated from distilled water wash fluids and analyzed by SDS-PAGE was stained for carbohydrate using periodic acid-Schiff (PAS) staining. Although S-protein itself failed to stain, it did appear that very faint LPS bands exhibiting a large degree of size heterogeneity could be detected on these gels. In order to confirm the presence of LPS, the concentrated distilled water wash fluids were assayed for KDO, a component of *A. vinelandii* LPS (Olin and Warner, 1967); a level of 0.8 µg KDO/mg protein was found. Further, it was estimated that distilled water washing released only 1% of the total cellular LPS. Similarly, the amount of carbohydrate (due to the O-side chains of the LPS) detected in the concentrated wash fluids was also low (30 µg/ mg protein) when assayed by both the phenol sulfuric and anthrone methods (Herbert *et al.,* 1971).

Attempts at reassembling the S-layer of *A. vinelandii in vitro*

A number of experiments were performed in which S-protein isolated from whole cells and dialyzed against distilled water, was mixed with distilled water washed cells in various proportions, concentrations, pH levels, divalent cation concentrations and buffer types but in no case was the reattachment of S-protein to the cell surface observed; S-protein prepared from whole cells appeared totally inactive. In contrast, incubation of S-protein extracted from OMV, with its "parent" outer membrane fragments, in the presence of mono- or divalent cations, produced reattachment of S-protein to the OMV in all cases. The level of reattachment was dependent upon the ionic strength of the suspending medium and the valence of the cation present (Fig. 2-8). Be^{2+} caused all detectable protein to reattach to the OMV; all other cations supported 80-85% reattachment of S-protein to the OMV at an ionic strength of 1×10^{-2}. Both monovalent and divalent cations were effective in reattachment at an ionic strength of 5×10^{-3}, but a reduction in ionic strength to 5×10^{-4} rendered the monovalent cations considerably less effective while the effectiveness of the divalent cations (Be^{2+} excepted) was only slightly impaired. At an ionic strength of 5×10^{-5} neither monovalent nor divalent cations caused a significant reattachment of S-protein to the OMV. OMV with attached S-protein were examined in the electron microscope after negative staining with 1% ammonium molybdate, pH 7 (Buckmire and Murray, 1976; Thorne *et al.*, 1975) and after freeze-etch treatment but

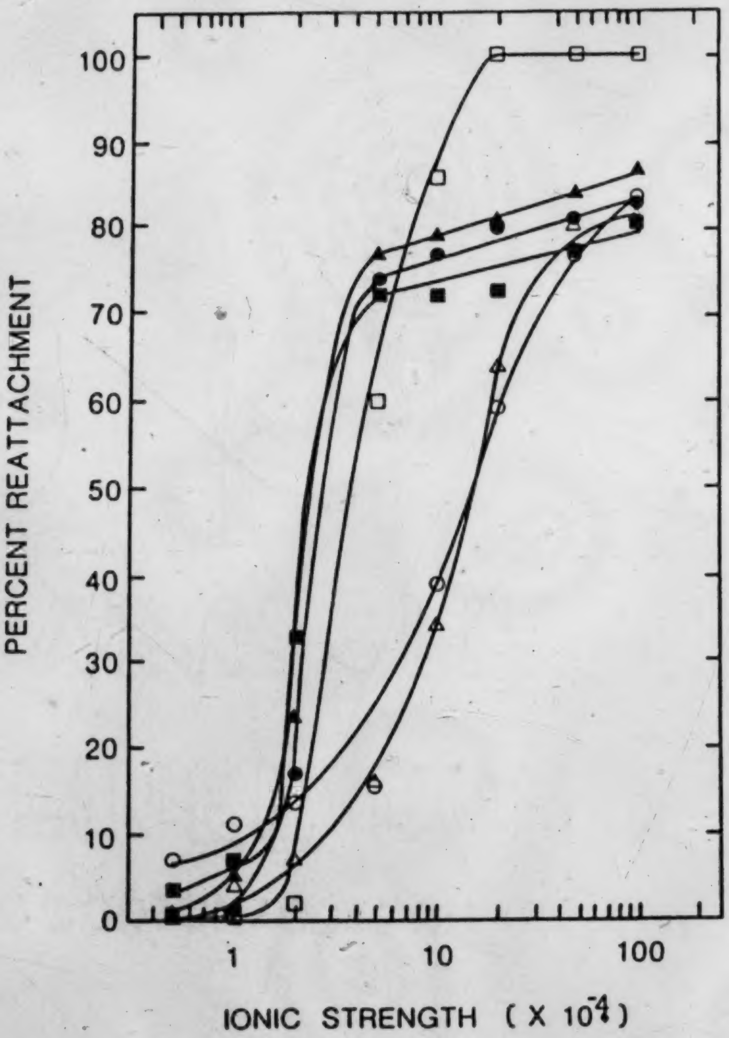

Fig.2-8. Effect of ionic strength on reattachment of S-protein to outer membrane vesicles. The cations used were Na^+ (△), K^+ (○), Mg^{2+} (▲), Be^{2+} (□), Ca^{2+} (●) and Sr^{2+} (■). Ionic strength (μ) was calculated using the following formula: $\mu = 1/2\sum c_i z_i^2$, where c = concentration (molar) and z = charge of each ionic species, i .

a regular surface layer nor isolated subunits were observed. By monitoring the polypeptide composition of the OMV (Fig. 2-9) it was clear that it was reattachment of S-protein which was being measured in the experiment shown in Figure 2-8. Figure 2-9 illustrates the results obtained using Ca^{2+}, Mg^{2+} and K^{+}. Similar results were obtained with Sr^{2+}, Be^{2+} and Na^{+}.

In an effort to confirm that S-protein was actually attaching to the OMV and not simply forming microaggregates of self-assembled protein which cosedimented with the OMV (Thorne *et al.*, 1975) the experiment depicted in Figure 2-8 was repeated without the addition of OMV to the S-protein cation mixtures. After a 2 h incubation the mixtures were centrifuged at 100,000 g for 30 min and the surface of the supernatant fluid was sampled (without decantation); no reduction in protein concentration was detected. In a second experiment, mixtures of cation and S-protein, with and without OMV, were loaded onto companion two-step sucrose gradients composed of a small (1 ml) 72% step and a large (8ml) 15% step. The gradients were centrifuged at 200,000 g for 90 min (Page and von Tigerstrom, 1982) and the material at the interface between the 15% and 72% layers was collected and analyzed by SDS-PAGE. S-protein could be recovered from the interface only when OMV were present in the S-protein-cation mixtures.

The specificity of the S-protein outer membrane reaction was tested by substituting cytoplasmic membrane vesicles (CMV) for outer membrane vesicles in the reaction mixtures. This experiment (Table 2-2) showed that S-protein also attached to a cytoplasmic membrane surface. Although attachment levels were 20-30% lower for CMV there was no way of determining whether the amount of exposed surface was the same for the OMV and CMV. In the reaction mixtures, equivalent amounts of membrane protein (either OMV or CMV) were mixed with a solution of S-protein. The ratio of S-protein to outer membrane protein was that normally found in the native cell wall but this did not necessarily indicate anything about the amount of surface available for S-protein binding in the CMV preparation.. Nevertheless, significant amounts of S-protein did bind to CMV. Reattachment of S-protein to OMV was also mediated by 8 mM Tris/HCl, pH 7.8 buffer (Fig. 2-9; lane 2).

A puzzling aspect of all the experiments described above was that some level of precipitation of S-protein by Ca^{2+} and Sr^{2+} was expected (Page and Doran, 1981); this was not observed. These experiments led to a reinvestigation of the precipitation of S-protein by Ca^{2+} and Sr^{2+}.

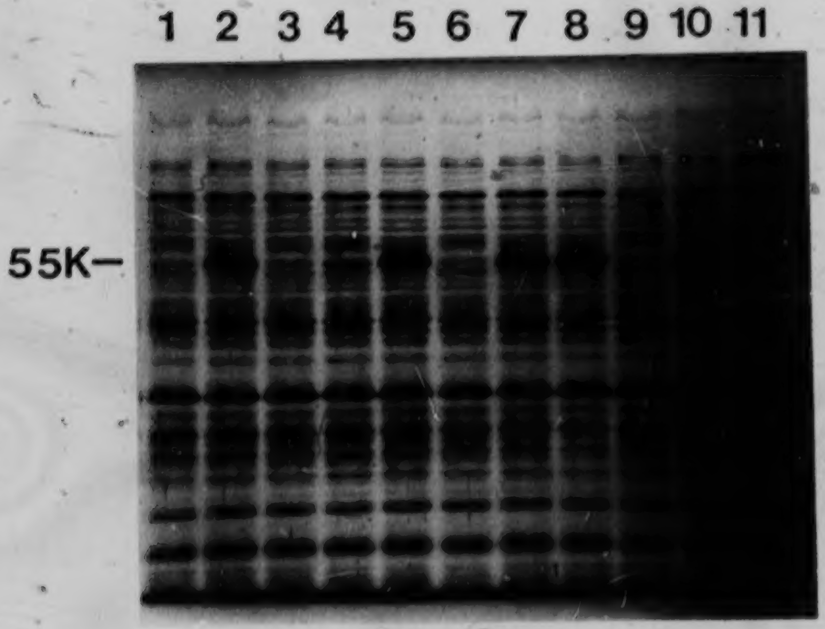

Fig.2-9. Reattachment of S-protein to outer membrane vesicles monitored by SDS-PAGE. Distilled water extracted outer membranes (lane 1) were incubated with S-protein and 8 mM Tris/HCl, pH 7.8 (lane 2) or K^+ (lanes 3 to 5), Ca^{2+} (lanes 6 to 8) and Mg^{2+} (lanes 9 to 11) at ionic strengths of 5×10^{-5} (lanes 3, 6, 9), 5×10^{-4} (lanes 4, 7, 10) or 5×10^{-3} (lanes 5, 8, 11).

Table 2-2. Mono- and divalent cation-mediated reattachment of S-protein to outer and inner membrane vesicles.

Cation	Reattachment (%)[1]	
	Outer membrane	Inner membrane
Na^+	78	31
K^+	73	23
Be^{2+}	100	100
Mg^{2+}	76	55
Ca^{2+}	78	51
Sr^{2+}	78	46

[1] Outer or inner membrane vesicles (1.5 mg protein) were mixed with 300 µg freshly extracted S-protein and monovalent (5 mM) or divalent (2 mM) cations for 2 h at room temperature. The membrane vesicles were removed by centrifugation at 100,000 g for 30 min, the reduction in S-protein concentration was measured and the level of attachment was calculated.

Precipitation of S-protein with divalent cations

S-protein (1 mg/ml) isolated from distilled water washing of whole cells was incubated with various concentrations of $CaCl_2$ between 0-100 mM. After 2 h the solution was centrifuged for 5 min in an Eppendorf centrifuge and the reduction in protein concentration of the supernatant was measured (Fig. 2-10). Approximately 80% of the protein could be precipitated when a concentration of $CaCl_2$ in excess of 20 mM was employed; further increase in the $CaCl_2$ concentration had no effect. The specificity of the reaction was examined by incubating S-protein with 20 mM concentrations of the chloride salts of Ca^{2+}, Ba^{2+}, Mg^{2+}, Sr^{2+}, Tb^{3+} and the nitrate salts of Be^{2+} and Ca^{2+} (Table 2-3). Neither Ba^{2+} or Mg^{2+} caused precipitation of the protein. However, the Ca^{2+} analogues Tb^{3+} and Sr^{2+} did cause precipitation but were not as effective as Ca^{+2}. Further, the protein was precipitated by Ca^{2+} regardless of its companion anion (Cl^- or NO_3^-). Interestingly, Be^{2+} was the most effective divalent cation for the precipitation of S-protein.

When S-protein solutions were subjected to dialysis prior to performing precipitation experiments, the protein lost all ability to precipitate with Ca^{2+}. Initially, it was thought that the protein had become denatured during dialysis against distilled water, but this seemed unlikely since it was originally isolated in highly dilute form in distilled water, spent considerable time during concentration in distilled water, and was subjected to a number of freeze-thaw cycles. Although the dialysis tubing was boiled in Na_2CO_3/EDTA prior to use, the possibility was investigated that some contaminant from the dialysis tubing (possibly a protease) rendered S-protein inactive. To test this idea, one sample of S-protein was dialyzed against distilled water and a duplicate sample was combined with a piece of dialysis tubing. Only the dialyzed sample lost the ability to precipitate with Ca^{2+}. Similarly, S-protein subjected to gel filtration on a Sephadex G-25 column lost the ability to precipitate with Ca^{2+}. These results indicated that some low molecular weight species was required for S-protein precipitation by Ca^{2+}. In a final experiment, a sample of the concentrated distilled water wash fluid was subjected to ultrafiltration using the Amicon micropartition system in order to separate any protein from the wash fluids. Protein assay confirmed that the ultrafiltrate contained no detectable protein, yet when the protein-free ultrafiltrate was made 20 mM with $CaCl_2$ a large flocculant precipitate formed in the absence of S-protein. Secondly, when samples of S-protein (1 mg/ml) desalted either by dialysis or gel filtration were mixed with the ultrafiltrate in a 1:1 ratio followed by the

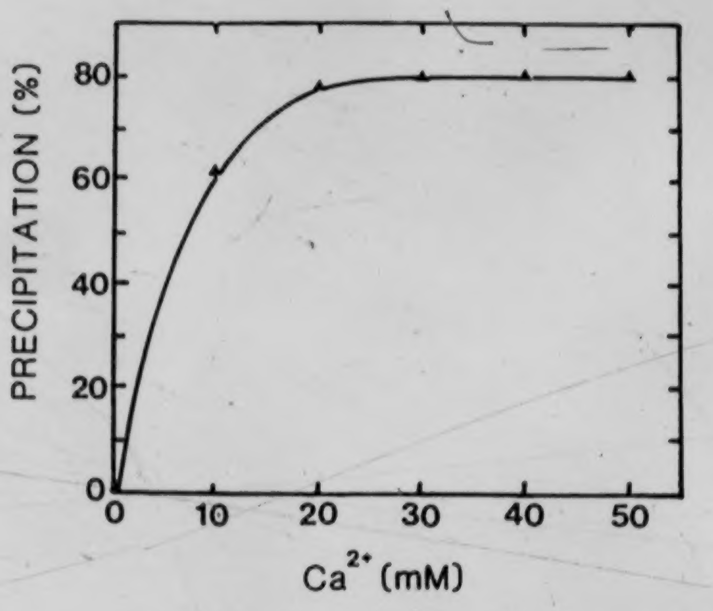

Fig.2-10. Effect of Ca^{2+} concentration on the precipitation of S-protein. Calcium chloride was added to S-protein solutions (1 mg/ml) and incubated for 1 h at room temperature. The resulting precipitate was removed by centrifugation and the level of precipitation was estimated by protein assay.

Table 2-3. Precipitation of S-protein with polyvalent cations[1].

Cation	Precipitation (%)
Ba^{2+}	7
Be^{2+}	100
Ca^{2+}	91
Mg^{2+}	2
Sr^{2+}	52
Tb^{3+}	79

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[1] Polyvalent cations (20 mM) were incubated with S-protein (1mg/ml) for 2 h at room temperature in Eppendorf centrifuge tubes. Any resulting precipitate was collected by centrifugation and the reduction in soluble S-protein concentration was assayed.

of Ca^{2+} (20 mM), S-protein regained its ability to precipitate to a level of 80% of a crude (undesalted) sample mixed in a 1:1 ratio with distilled water. All of these experiments indicated that precipitation of S-protein by Ca^{2+} was a secondary event. Addition of 20 mM Ca^{2+} or Sr^{2+} to $BBPO_4$ alone resulted in precipitate formation while addition of a similar level of Mg^{2+} did not. Therefore, it was likely that trace contamination of the distilled water wash fluids with PO_4^{3-} produced insoluble salts upon addition of Ca^{2+} and Sr^{2+} which subsequently adsorbed S-protein giving the impression of specific precipitation by these cations. Since Mg^{2+} did not form insoluble phosphate salts, no 'precipitation' of the protein was observed.

2.4 Discussion

A. vinelandii UW1 possesses an S-layer of tetragonally arranged protein subunits superficially similar to S-layers observed on many other bacteria (Sleytr and Messner, 1983). Cells grown under iron-sufficient conditions require a more rigorous washing regime than iron-limited cells (Doran, 1983) to expose the S-layer for visualization by freeze-etch electron microscopy. The reasons for this are not clear. Washing and heating the cells before freeze-etching possibly removed minor amounts of capsular material thought to be produced by strain OP (Page, 1984) which otherwise may have collapsed over the S-layer during etching (Sleytr and Messner, 1983). However, iron-limitation has been reported to enhance capsule production (Jarman *et al.*, 1978) which does not correlate with the greater difficulty in exposing the S-layer in iron-sufficient cells. Similarly, problems in visualizing the *Caulobacter crescentus* surface array by both negative staining and freeze-etching have been noted by Smit *et al.* (1981). These workers also considered the possibility of interfering capsular material.

Although its reported theoretical molecular weight, derived from amino acid analysis is 65,000 (Schenk and Earhart, 1981), the principle component of the S-layer is a protein which ranges from 55,000-60,000 apparent molecular weight on SDS-PAGE. In my hands, an apparent molecular weight of 55,000 is consistently obtained, the value reported by Page and von Tigerstrom (1982). Three other additional polypeptides in trace amounts appear to be coextracted from whole cells with S-protein. These proteins do not appear to be outer membrane proteins since they do not possess the same mobility as any major outer membrane protein (see Fig. 2-3) and the level of LPS release from the outer membrane by distilled water washing does not indicate any serious disruption has occurred. The nature and origin of these species awaits further investigation.

The radioiodination behaviour of S-protein does not appear consistent with its known surface localization. Although it completely envelopes the cell, it is only weakly labelled under conditions which clearly label integral outer membrane proteins. This result can be explained by considering differing accessibilities of tyrosine residues to iodination (Concino and Goodgal, 1981; Markwell and Fox, 1978). The tyrosine residues labelled under low reagent concentrations are probably those which interact most intimately with the environment. S-protein is labelled weakly under these conditions indicating some of its tyrosine residues are of this type. On the other hand, when high reagent concentrations are used S-protein becomes intensely labelled, while most integral outer membrane proteins fail to undergo the same degree of further radioiodination. This indicates that S-protein contains additional tyrosine residues which are more accessible than those of integral outer membrane proteins. These residues are probably located within tightly folded regions of the protein and are only accessible at high ^{125}I and Iodo-Gen levels. At the same time, integral outer membrane proteins fail to undergo the same degree of labelling because the majority of their polypeptide chains are buried in the outer membrane which provides an effective barrier to iodination (Loeb and Smith, 1983). When the exposure time for an autoradiograph is chosen based on the S-protein band, labelled integral outer membrane protein bands are almost undetectable. The appearance of a radioiodination pattern showing only the S-layer protein of *Aeromonas salmonicida* led Kay *et al.* (1981) to suggest that the S-layer of this organism was external. The results presented here suggest that such conclusions should be drawn with caution. The labelling of relatively accessible amino acid residues in an S-layer protein accounting for a large proportion of total envelope protein may mask the fact that other surface exposed proteins are also being labelled because exposures of autoradiographs adequate to visualize S-layer proteins may not detect less abundant species.

The S-protein of *A. vinelandii* has been reported to be a glycoprotein (Schenk, 1978; Page and Doran, 1981) due to its staining reaction on SDS gels; this could not be confirmed in the present study. Schenk (1981) used the Alcian blue staining method of Wardi and Michos (1972) while Page and Doran (1981) used the PAS method of Page and Stock (1974). The staining method of Wardi and Michos (1972) was not designed for use with SDS-gels and it has been subsequently found that this compound will stain any protein (not just glycoproteins) depending on whether the sodium dodecyl sulfate used is contaminated with sodium tetradecyl sulfate and the rigor with which SDS gels are washed before application of the stain (Kuniciki *et al.*, 1981). On the other hand, the problem with the data of Page and Doran (1981) may have due to a failure to destain the PAS stained gels effectively. It is therefore unlikely S-protein possesses a major carbohydrate moeity.

The release of the *A. vinelandii* S-protein by distilled water extraction alone is unique. The removal of other S-layer proteins from isolated cell walls usually requires either urea, guanidine/HCl, chelating agents, detergents, pH extremes or competing cations (Koval and Murray, 1984). Perhaps the most comparable treatment to that used for *A. vinelandii* is that used to release the S-protein from *Acinetobacter* 199A (Thornley *et al.,* 1974). This S-protein is released by sequential extraction with EDTA (or EGTA) and distilled water. During the EDTA extraction, LPS is released from the outer membrane but the protein remains cell wall associated. The subsequent distilled water extraction releases the S-protein in almost pure form. This treatment probably involves chelation of essential divalent cations followed by a reduction in ionic strength (provided by the EDTA itself) with distilled water, leading to a release of the protein. A similar mechanism could be involved in the release of the *A. vinelandii* S-protein. Page and Doran (1981) reported that S-protein, isolated from whole cells, was moderately acidic (pI 5.1). This suggests that salt bridging could be at least partially involved in its attachment to the cell surface. In the case of *A. vinelandii,* exposure of the cell to distilled water at an elevated temperature, may be sufficient to 'leach out' any divalent cations. An argument against this idea is that preincubation of the cells in Burk buffer (Protocol #2) rather than distilled water (Protocol #1) apparently makes the protein easier to wash from the cell surface. Because the distilled water had a pH of 5.2, H_30^+ displacement of essential cations could be the mechanism of S-protein release. However, during extraction of S-protein from outer membrane fragments, rapid neutralization of the distilled water by residual Tris buffer occurs, casting doubt on this hypothesis. Distilled water washing has been reported to completely remove S-protein from the cell surface (Schenk and Earhart, 1981). Although this impression is given by following the polypeptide composition of the outer membrane, cell surface radioiodination indicates some S-protein remains. However, the amount of residual S-protein is small when compared to the amounts of S-protein naturally present on the cell surface and should not hinder the use of whole cells as a template for reassembly.

Although there are a number of indications that divalent cations, specifically Ca^{2+}, are important to the structure of the surface array, it has not been possible using these species, to reassemble S-protein isolated from whole cells, back onto the cell surface under a variety of conditions. It is difficult to accept that the protein has been irreversibly denatured since the treatment used to isolate the *A. vinelandii* S-protein is the most benign protocol found in the S-layer literature (Koval and Murray, 1984). S-proteins requiring divalent cations for assembly are commonly isolated using urea and guanidine/HCl. Once these agents are removed the surface layer proteins can reassemble into a regular array provided suitable conditions are provided (Kist and Murray, 1984; Koval and Murray, 1983; Thornley *et al.*, 1974). It is possible for the *A. vinelandii* system that suitable reassembly conditions have not been identified, but this seems unlikely. The most probable explanation is that S-protein, as isolated, is incapable of reassembling into a regular array. Because the specific precipitation of the protein by Ca^{2+} ions was found to be an artifact, there is really no objective method of determining whether the S-protein isolated from whole cells has been irreversibly denatured during isolation beyond its ability to reassemble *in vitro*. Although the specific precipitation of the protein by Ca^{2+} and its analogues seemed to be linked to the necessity for Ca^{2+} in the growth medium for regular array formation (Doran, 1983) this was a nasty coincidence. Despite the amount of effort expended in optimizing the yield of S-protein from whole cells, determining purity and the investigation of conditions for *in vitro* reassembly, the use of S-protein isolated from whole cells was abandoned in favour of that released from outer membrane fragments.

Schenk and Earhart (1981) reported that S-protein from *A. vinelandii* copurified with outer membrane fragments, prepared using EDTA, only if the mixture was subsequently made 2 mM with any of a number of divalent cations. Although the significance of this observation was unclear, because of the extensive damage to the outer membrane caused by EDTA (Cho *et al.*, 1974), it did suggest that S-protein released from outer membrane fragments could be active in divalent cation-mediated reassembly of the S-layer. To test this hypothesis, outer membranes were recovered by the method of Page and von Tigerstrom (1982) which involved no EDTA treatment. S-protein was released by distilled water extraction and assayed for the ability to reattach to the outer membrane in the presence of mono- and divalent cations. While reattachment could be documented, no regular array formation was detected by electron microscopy. However in this instance, a problem may exist with the template surface rather than S-protein. The limited experiments with cytoplasmic membrane vesicles have shown that S-protein will attach to a quite heterologous surface; this casts doubt on the usefulness of isolated cell walls as a template surface for *in vitro* reassembly. If the membrane vesicles are of inverted topology (Barnes *et al.*, 1977), altered permeability (Irvin *et al.*, 1981) or incompletely sealed (Nakae and Nikaido, 1975) they could present both the inner and outer surfaces of the outer membrane for S-protein attachment. The competition for S-protein, by the inner surface of the outer membrane, may possibly disrupt the formation of the regular array in its natural location, the outer leaflet of the outer membrane. The logical extension of the findings reported here, would be to develop a reassembly system using: (1) whole cells as a template surface and (2) S-protein isolated from outer membrane fragments as the source of "biologically active" surface array protein.

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3. Characterization of the surface layer protein from *Azotobacter vinelandii*

3.1 Introduction

Surface layers of regularly arranged protein subunits are now recognized as common features of eubacterial cell walls (Sleytr and Messner, 1983). Distributed widely among both gram-positive and gram-negative eubacteria, these arrays are noncovalently attached to the surface of the outer membrane or the peptidoglycan sacculus in linear, tetragonal or hexagonal formats, forming in some cases the primary barrier between the cell and the environment. This regularity of structure has been exploited for ultrastructural analyses through electron microscopy and image processing methods (Beveridge, 1981; Sleytr and Glauert, 1982). Despite the detailed information concerning S-layer ultrastructure, the biochemical analysis of these layers has only been preliminary. A notable exception to this generalization is the study of Phipps *et al.* (1983) which outlined the purification and characterization of the S-protein of *Aeromonas salmonicida.* Recently, the entire S-protein genes of *Caulobacter crescentus* (Smit and Agabian, 1984) and *Deinococcus radiodurans* (Peters and Baumeister, 1986) were cloned as well as a large portion of the S-protein gene of *Bacillus brevis* 47 (Tsukagoshi *et al.*, 1984). This should facilitate the further biochemical analysis of these surface array proteins.

The S-layer protein of *Azotobacter vinelandii* (Bingle *et al.*, 1984) would appear to be a useful model system for the study of structural aspects of a surface array protein since it can be released from the cell surface with distilled water, a reagent unlikely to induce radical perturbations in its structure when compared to the reagents used to solubilize other S-layer proteins (Koval and Murray, 1984). However, conditions for its reassembly into a regular array have not been elucidated, and distilled water extracts appear to contain three minor contaminating proteins. A preliminary characterization of the protein constituting the *A. vinelandii* S-layer (S-protein) was made by Schenk and Earhart (1981). However, this study used S-protein washed directly from heavily encapsulated whole cells and S-protein prepared in this manner apparently does not self-assemble (Chapter 2). This study has employed both a capsule-negative strain of *A. vinelandii* and isolated outer membrane

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fragments instead of whole cells as a primary source of S-protein. A significant aid in the purification of the *A. vinelandii* S-protein is that it can be released from outer membrane fragments by a single distilled water extraction (Schenk *et al.*, 1977).

This chapter outlines: (1) the development of a system for the *in vitro* reassembly of the *A. vinelandii* S-layer, (2) a purification scheme to separate S-protein from the three minor contaminating proteins found in distilled water extracts, (3) a biochemical characterization of purified S-protein and (4) the interaction of purified S-protein with divalent cations.

3.2 Materials and Methods

Bacterial strains and growth conditions

A. vinelandii UW1 was grown in Burk buffer containing ammonium acetate and glucose as previously described (Bingle *et al.*, 1984; Chapter 2). For ^{35}S-labelling of whole cells, all sulfate salts were replaced with chloride salts and Na_2SO_4 was included at a concentration of 0.2 mM. $^{35}SO_4^{2-}$ was added as H_2SO_4 to a specific concentration of 1mCi/100 ml or 0.5 mCi/100 ml (1 Ci=37 GBq). $BBPO_4$ refers to the potassium phosphate buffer of Burk buffer (4.6 mM K_2HPO_4/1.6 mM KH_2PO_4, pH 7.2).OFeBB refers to iron-limited Burk buffer (Page and von Tigerstrom, 1982; Chapter 2).

Isolation of S-protein

Outer membranes were prepared from approximately 16 g wet weight of cells essentially by the method of Page and von Tigerstrom (1982). Briefly, the cells were resuspended in 10 mM Tris/HCl, pH 7.5, containing 10% (w/v) sucrose and disrupted using the French pressure cell. Outer membranes were separated from cytoplasmic membrane and poly-ß-hydroxybutyrate granules (PHB) on sucrose step gradients. For larger scale isolation of outer membrane fragments, concentration steps forming the upper part of the gradient were reduced in volume or eliminated to accommodate larger loads of material; this did not reduce the resolution of the gradient. All sucrose solutions were made in 10 mM Tris/HCl, pH 7.5 and all manipulations were conducted on ice unless otherwise specified. Outer membrane material collecting below the PHB band was recovered and diluted 5-fold with 10 mM Tris/HCl, pH 7.5. This suspension was dispensed into polycarbonate centrifuge tubes and centrifuged at 4°C for 1 h at 100,000 g. Each of the resulting outer membrane pellets (each approximately 3 mg protein) was extracted with 1 ml of distilled water at room temperature by agitating with a vortex mixer at high speed. Outer membrane fragments were then removed by centrifugation at 100,000 g for 30 min at 4°C. The resulting S-protein solution contained approximately 1 mg/ml protein when assayed by the Markwell *et al.* (1978) modification of the Lowry procedure using bovine serum albumin (BSA) as a standard. Finally, the buffer concentration was brought to a final concentration of 10 mM through the addition of 1 M Tris/HCl, pH 7.5 and the solution was routinely stored at -20°C prior to further use. S-protein was further purified at 4°C on Sephadex G-100 Superfine using 10 mM Tris/HCl, pH 7.5 as the elution buffer. S-protein eluting in the same fractions as BSA (molecular weight=66,000) was pooled, concentrated and used in all experiments unless otherwise specified.

Concentration and desalting of S-protein preparations

S-protein solutions were concentrated by ultrafiltration using an Amicon PM-10 ultrafiltration membrane (Amicon Corp., Oakville, Ont.) which had a molecular weight cut-off of 10,000. Small volumes of S-protein solutions were concentrated by centrifugation with the Amicon ultrafiltration-micropartition system (YMT membrane; molecular weight cut-off, 10,000). Centrifugation was interrupted at 5 min intervals to dislodge any protein that may have collected on the membrane and to ensure concentration did not proceed to complete dryness. Desalting of S-protein preparations was accomplished using a small Bio-Gel P-6DG (BioRad Laboratories, Mississauga, Ont.) gel filtration column (1.5 X 11 cm). This column was also used to exchange Tris buffer in S-protein samples for either distilled water or $BBPO_4$ buffer.

Rate zonal centrifugation

Linear glycerol gradients (12 ml) of 6-30% (v/v) in the appropriate buffer were prepared by layering solutions differing in concentration by 2% (v/v) glycerol in Beckman SW40 ultraclear centrifuge tubes. The gradients were held at 4°C for 18 h to permit diffusion to abolish the interfaces and establish linearity. S-protein samples (200 µl; 0.8 mg protein) were carefully added to the top of the gradients and centrifugation was performed at 40,000 rpm (200,000 g) for 21.5 h at 4°C. Twenty-four 35-drop fractions were collected by puncturing the bottom of the tube with a 21-gauge needle.

Circular dichroism (CD) measurements and ultraviolet (UV) absorption spectra

CD spectra were determined using a Cary 60 spectropolarimeter in conjunction with a model 6001 CD attachment according to Oikawa *et al.* (1968).

The percent α-helix, ß-sheet and random coil configurations were calculated using the experimental mean residue ellipticities at 213 nm, 222 nm and 225 nm using the following equations from Chen *et al.* (1972):

$$[\theta]_{213} = -26.4(f_{\alpha}) - 9.68(f_{\beta}) - 3.50(f_{RC})$$

$$[\theta]_{222} = -31.5(f_{\alpha}) - 2.67(f_{\beta}) - 2.78(f_{RC})$$

$$[\theta]_{225} = -30.0(f_{\alpha}) - 2.00(f_{\beta}) - 3.38(f_{RC})$$

where $[\theta]$ represents the mean residue ellipticity in (degrees cm^2 per decimole) x10^{-3} and f_{α}, f_{β} and f_{RC} represent fractions of α-helix, ß-sheet and random coil configurations respectively. The mean residue molecular weight of 104 was calculated using the theoretical molecular weight from the amino acid analysis. The absolute protein concentration was calculated based on the modified Lowry procedure of Markwell *et al.* (1978) using BSA as a standard. A conversion factor of 0.565 derived from the total amino acid analysis was used, i.e., [absolute] = 0.565 [relative]. The above set of simultaneous equations was solved using the matrix algebra capabilities of the APL computer language.

UV absorption spectra of S-protein solutions were determined using a Perkin Elmer Lambda 3 spectrophotometer.

Amino acid analysis

S-protein extracted from the outer membrane with distilled water was desalted, lyophilized, and hydrolyzed in 6 N HCl for 24, 48, 72 and 96 h at 110°C. The evaporated hydrolysates were resuspended in 0.2 M sodium citrate, pH 2.2, containing 1% phenol and 0.5% (v/v) thiodiglycol. Amino acid analysis was performed using a Dionex-D500 amino acid analyzer. Serine and threonine composition were estimated by extrapolating to zero time. Valine values were taken from hydrolysis periods in excess of 24 h. Tryptophan content was estimated spectrophotometrically by the method of Edelhoch (1967).

Reassembly assay

Cells were washed with distilled water to remove the native S-layer as described by Bingle *et al.*, 1984). Distilled water washed cells were resuspended to an optical density at 620 nm of 13 in 2 ml of an S-protein solution in distilled water (220 μg/ml protein) containing 1 mM $CaCl_2$ and/or $MgCl_2$. The suspension was shaken slowly (150 rpm) for 2 h at 30°C and the cells were pelleted at top speed in an IEC clinical centrifuge for 10 min at room temperature. The amount of S-protein that remained in the supernatant was assayed (Markwell *et al.*, 1978), and if attachment to the cells occurred, the cells were examined by freeze-etch electron microscopy for evidence of regular array formation.

Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted as previously described (Bingle *et al.*, 1984; Chapter 2). Samples were boiled for 7 min in SDS-sample buffer containing 2% SDS, 10% glycerol and 5% ß-mercaptoethanol in 62.5 mM Tris/HCl, pH 6.8, before application to the gel. Nondenaturing gels were run in the absence of SDS and samples were not pretreated as described above. Isoelectric focussing of S-protein was performed in vertical slab polyacrylamide gels (7% acrylamide, 0.2% N,N'-methylenebisacrylamide) in the presence of 9.2 M urea using apparatus and conditions described by Page and Doran (1981). The pH gradient was formed using a mixture of pH 4-6 and 3.5-10 LKB ampholines present at a concentration of 1.6 and 0.4% (w/v) respectively. The pH gradient was determined by excising a lane from the gel and incubating slices of 0.5 cm in length in 2 ml of distilled water for 24 h. Gels were stained by a modification of the method of Fairbanks *et al.* (1971) described by Page and Doran (1981).

Freeze-etch electron microscopy

Freeze-etch electron microscopy of whole cells was performed as described previously (Bingle *et al.*, 1984; Chapter 2).

3.3 Results

Reassembly of S-protein onto the surface of distilled water washed cells

S-protein could be reattached to distilled water washed whole cells with a variety cations (Fig. 3-1). Monovalent cations were unable to support levels of reattachment comparable to those with divalent cations at an ionic strength of 5×10^{-3}. In the case of Mg^{2+}, Ca^{2+} and Sr^{2+} there was little increase in reattachment of S-protein afforded by increasing the concentration of these ions in excess of 0.5 mM which agreed well with the levels of two of these ions in the growth medium. In contrast to the other divalent cations, Be^{2+} promoted an enhanced reattachment of S-protein over the entire concentration range tested.

Whole cells with reattached S-protein were examined by freeze-etch electron microscopy for evidence of the reassembly of the regular array. It was not necessary to wash or heat these cells before freeze-etching to expose the regular S-layer. Distilled water-washed whole cells incubated with S-protein and 1 mM Mg^{2+}, Ca^{2+}, Sr^{2+} or OFeBB showed extensive areas of typical tetragonal array frequently covering the entire visible cell surface (Fig. 3-2). Be^{2+} did not promote regular array formation and the cell surface appeared to be free of visible S-protein subunits (Fig. 3-3). A control sample incubated in OFeBB in the absence of S-protein showed no regular array formation, indicating addition of S-protein was required. The lack of *de novo* synthesis of S-protein was confirmed by incubating distilled water washed cells with 1 mM Ca^{2+}, Mg^{2+}, Sr^{2+} or OFeBB for 2 h at 30°C with shaking, isolating the outer membrane, and extracting with distilled water. No protein was detected in excess of the trace amount released from a similarly extracted unincubated control.

Homogeneity of S-protein preparations

S-protein extracted directly from outer membrane fragments and used in the reassembly experiments, like that isolated from whole cells, was not a completely pure preparation and

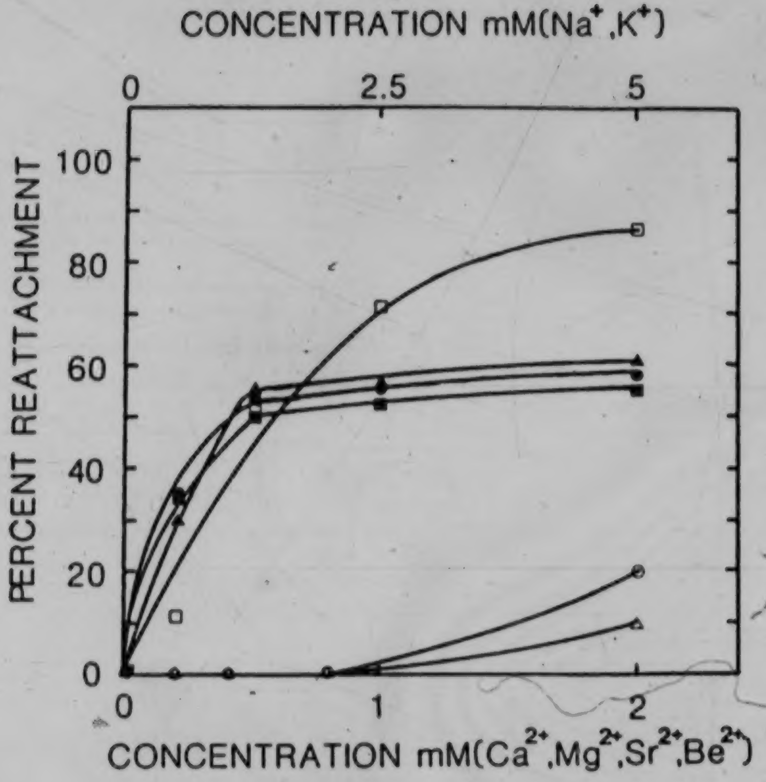

Fig. 3-1. Reattachment of S-protein to distilled water washed cells. Isolated outer membrane was used as a source of S-protein which was mixed with with whole cells at 30°C for 2 h in the presence of Na$^+$ (△), K$^+$ (○), Mg^{2+} (▲), Ca^{2+} (●), Sr^{2+} (■) or Be^{2+} (□). Monovalent cations were added to a concentration of 0.5, 1, 2, or 5 mM, while divalent cations were added to a concentration of 0.2, 0.5, 1 or 2 mM. Reattachment of S-protein was determined as described in Materials and Methods.

Fig. 3-2. Divalent cation (Mg^{2+}) mediated *in vitro* reassembly of freshly isolated S-protein onto the surface of distilled water washed *A. vinelandii* whole cells. Freeze-etch replica; Bar, 0.5 µm.

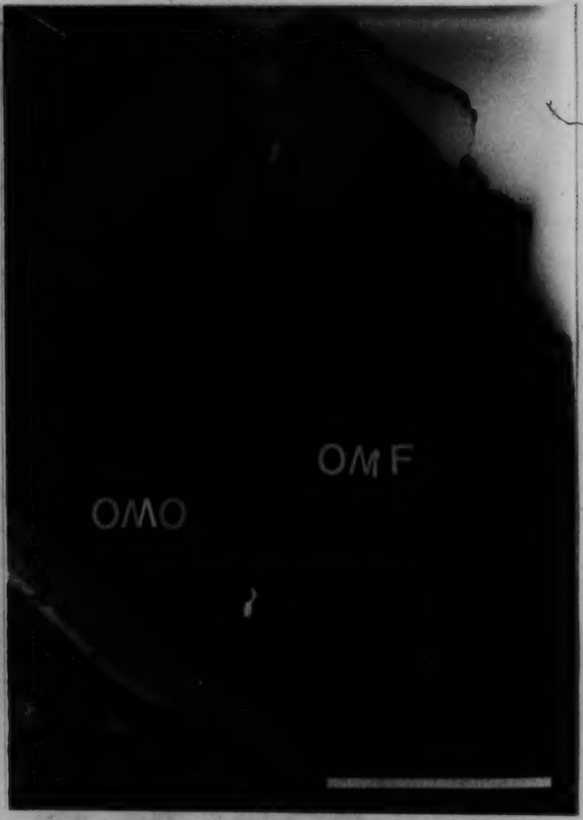

Fig. 3-3. Freeze-etch replica of distilled water washed *A. vinelandii* UW1 whole cells incubated with S-protein in the presence of 0.5 mM Be^{2+}. Bar, 0.5 µm.

contained three additional proteins of 45,000, 30,000 and 16,000 apparent molecular weight when analyzed by SDS-PAGE (Fig. 3-4: insert). The three smaller additional polypeptides could have represented unique protein species present as: (1) minor components of the S-layer, (2) contaminating outer membrane proteins either in a soluble form or associated with small outer membrane fragments or (3) possible proteolytic degradation products of S-protein.

If the three minor species represented unique proteins, in a soluble form or as outer membrane fragments, it should be possible to separate them from S-protein by gel filtration. When a distilled water extract of outer membrane was chromatographed on Sephadex G-75 Superfine, two peaks were observed (Fig. 3-4) a small void volume peak and a large peak eluting at the same position as BSA (molecular weight = 66,000). The column was capable of separating BSA from ovalbumin (OVA; molecular weight = 45,000) and therefore, if the 45, 30 and 16K bands were unique protein species, they should be absent from the main S-protein peak. Nevertheless, when the peak fraction was analyzed by SDS-PAGE, the three minor protein bands were present apparently unaffected by the chromatographic step (Fig. 3-4: insert). If the contaminating bands were present as small outer membrane fragments, they would be expected to elute at the void volume. It was therefore unlikely these species were present in a particulate form. While the possibility of proteolysis could not be completely ruled out, the integral nature of the molecular weights, i.e., multiples of approximately 15K was unusual. This fact coupled with the unusual property (for a bacterial S-protein) of significant quantities of cysteine (Schenk and Earhart, 1981) in the protein, suggested the possibility that boiling in SDS-sample buffer had failed to completely denature the *A. vinelandii* S-protein and that its apparent monomolecular weight was approximately 15,000 rather than 55,000-60,000. To test this hypothesis, the protein was heated in SDS-sample buffer (no ß-mercaptoethanol) for 5 min, at 10°C intervals between 20 and 100°C. The unexpected result (Fig. 3-5) was that the presence of the lower molecular weight species was enhanced between 20-40°C. At 40°C, multiple bands were evident extending from the 30K to 55-60K form. In addition, the lowest molecular weight form (16K) observed in these gels (here running at the dye front) was also intensified at 40°C. Increasing the incubation temperature to 100°C caused a reduction but never a complete elimination of these minor bands with a concomitant production of the 55-60K form. Thus, the presence of the 16K, 30K and 45K bands were artifacts which resulted from incomplete S-protein denaturation. Only one major band was seen when S-protein was electrophoresed under native conditions using the same gel-buffer system in the absence of SDS (Fig. 3.6; lane 1).

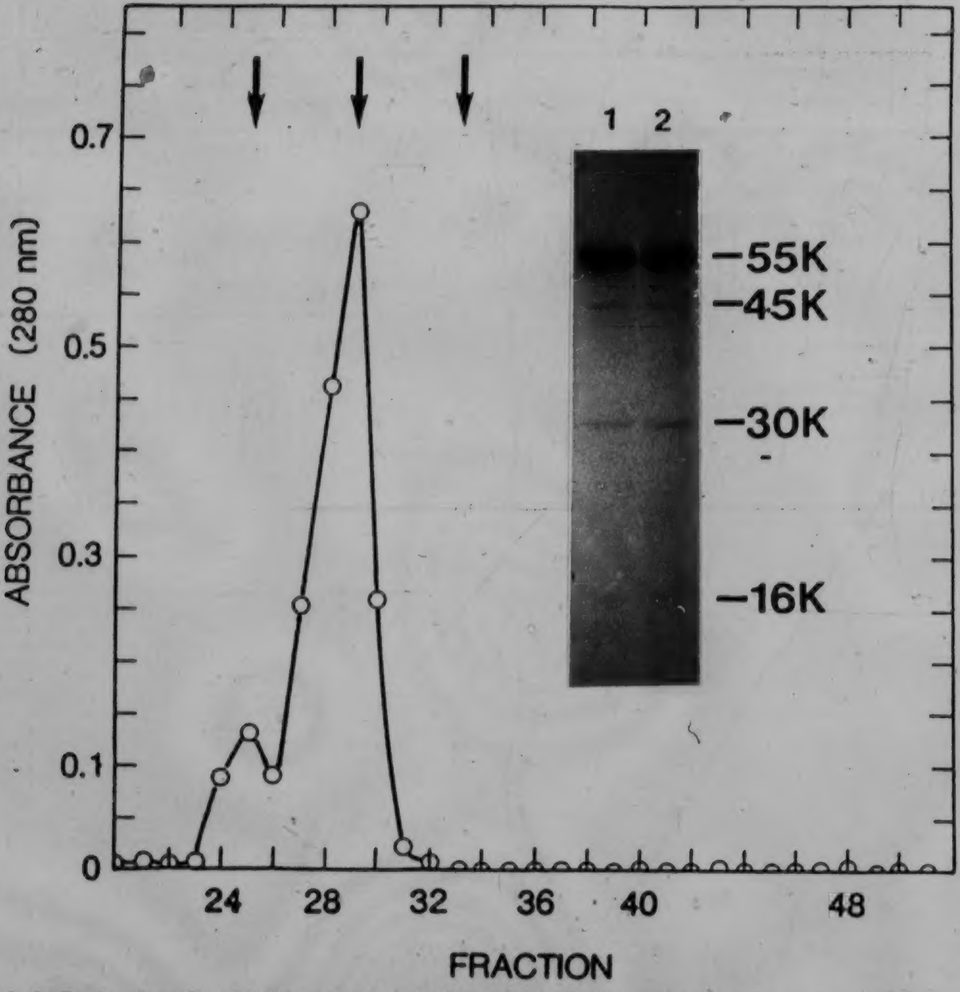

Fig. 3-4. Sephadex G-100 Superfine gel chromatography of S-protein extracted from outer membrane fragments with distilled water. The column (2.6 X 41 cm) was equilibrated at 4°C with 10 mM Tris/HCl, pH 7.5 and eluted with the same buffer at a flow rate of 10 ml/h collecting 2.8 ml fractions. Arrows indicate elution position of Blue Dextran (left), BSA (middle) and OVA (right). Inset: SDS-PAGE of S-protein preparations; lane 1, crude outer membrane extract before chromatography; lane 2, S-protein eluting in fraction 29.

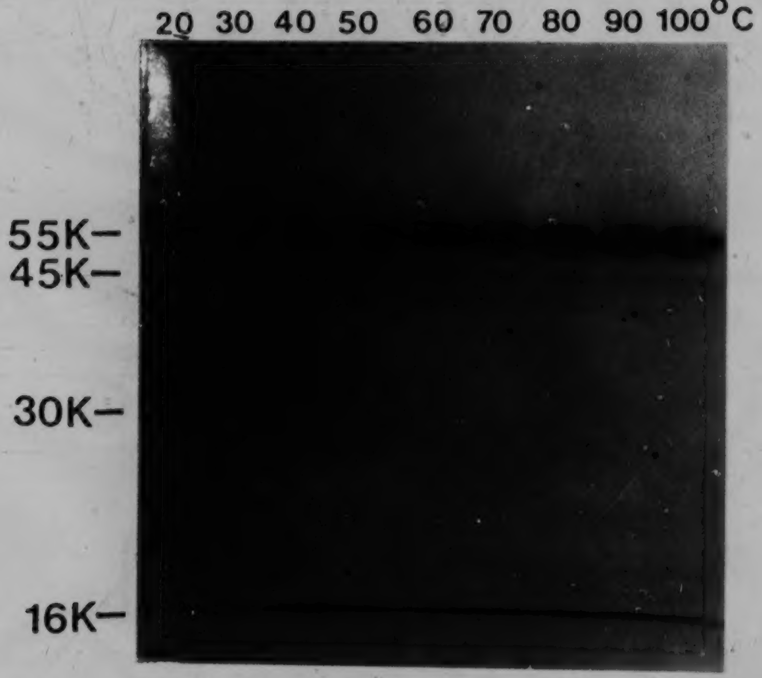
20 30 40 50 60 70 80 90 100°C
55K–
45K–
30K–
16K–

Fig. 3-5. Effect of treating S-protein at different temperatures in SDS-sample buffer. Samples (20 μg protein) were heated for 5 min at the temperatures indicated before application to the gel.

Fig. 3-6. Non-denaturing discontinuous gel electrophoresis of S-protein released directly from outer membrane fragments by distilled water extraction. Lanes: 1, untreated S-protein; 2, S-protein treated with 1% Sarkosyl; 3, S-protein treated with 10 mM EDTA.

Purity of S-protein preparations

The near-UV absorption spectrum of S-protein released directly from outer membrane fragments by distilled water was significantly different from that of S-protein further purified by gel filtration on Sephadex G-100 Superfine (Fig. 3-7; a, c). While both possessed an absorption maximum at 278 nm and a minimum at 250 nm, the A_{280}:A_{260} ratio was significantly lower in the former preparation, indicating likely nucleic acid contamination. However, most of these impurities could be removed by passing the S-protein preparation over a Bio-Gel P-6DG desalting column (Fig. 3-7; b). Based on tabulations by Warburg and Christian (1941), the desalted preparation contained about 1% nucleic acid. KDO(2-keto-3-deoxyoctanoic acid) was detected at a level of 0.5 µg/mg protein in desalted S-protein preparations when assayed by the method of Keleti and Lederer (1974). Olin and Warner (1967) reported a KDO:lipopolysaccharide (LPS) ratio of 1.33 mmole/gram LPS for LPS extracted from *A. vinelandii* with cold phenol. This amounts to 33% by weight KDO which gives an LPS:S-protein ratio of 1.5 µg/mg protein. A value of 33% by weight KDO is high compared with other lipopolysaccharides, which rarely exceed 10% except in those molecules lacking O-side chains. Thus 1.5 µg/mg protein is likely to be an underestimate, perhaps by an order of magnitude. Nevertheless, desalted S-protein released from outer membrane fragments was present in a highly purified state contaminated by no more than 1-2% nucleic acid or 1-2% LPS.

Amino acid composition of S-protein

The amino acid composition of S-protein is presented in Table 3-1. The values presented are based on separate determinations using two different S-protein samples. The only sulfur containing amino acid detected was methionine. The protein contained a large proportion of hydrophobic amino acids (44%) in addition to 19% acidic amino acids and 7% basic amino acids. Although the analysis did not distinguish between glutamic acid and glutamine or between aspartic acid and asparagine, isoelectric focussing confirmed the acidic nature of S-protein (Fig. 3-8). One major isoelectric form was present (pI 4.4) surrounded by a number of minor bands ranging between a pI of 4.2-4.6. Based on an apparent molecular weight of between 55,000 and 60,000 determined by SDS-PAGE, a theoretical molecular weight of 60, 218 (577 residues) was calculated using molar ratios with respect to methionine. The spectrophotometric method of Edelhoch (1967) indicated

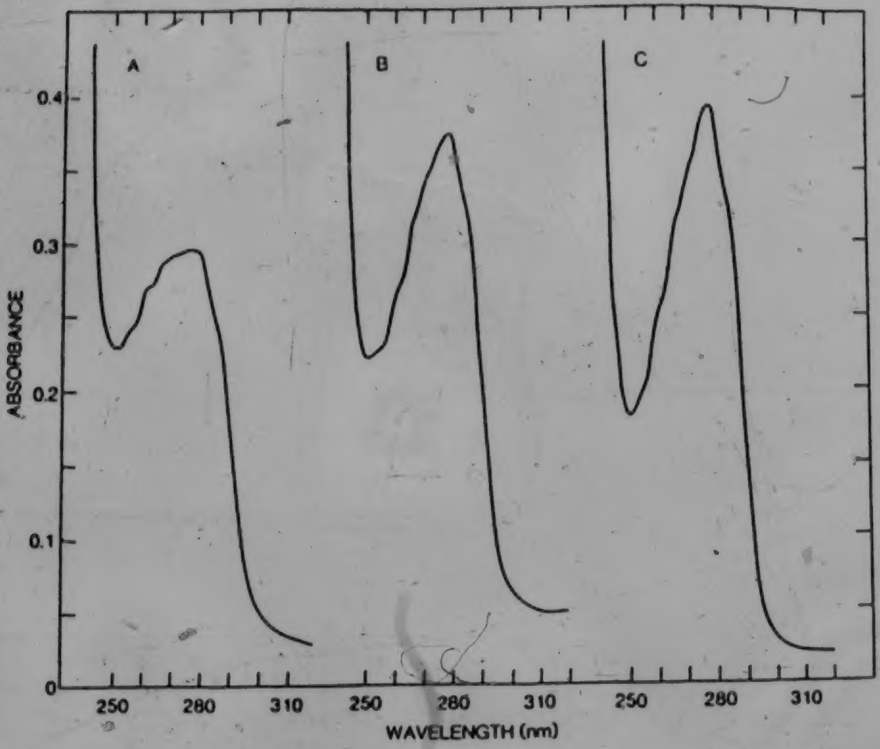

Fig. 3-7. UV absorption spectra of various S-protein preparations. (A) Crude S-protein released directly from outer membrane by distilled water extraction; (B) after desalting on Bio-Gel P-6DG; (C) after gel filtration chromatography on Sephadex G-100 Superfine (running conditions same as those described in Fig. 3-6).

Table 3-1. Amino acid analysis of the *A. vinelandii* S-protein.

Amino acid residue	mol %	
	This study	Schenk and Earhart (1981)
Asx	10.9	9.2
Glx	8.0	19.6
Thr	9.5	6.7
Ser	9.5	6.7
Pro	2.9	1.9
Gly	8.0	14.2
Ala	13.3	13.3
Val	8.3	5.5
Met	0.7	0.9
Ile	5.0	3.6
Leu	8.5	6.8
Tyr	2.4	0.9
Phe	5.4	3.3
His	NF[1]	0.4
Lys	4.9	3.4
Arg	2.1	1.0
Trp	0.5	ND[2]
Cys	NF	2.5

[1] Not found; [2] Not determined.

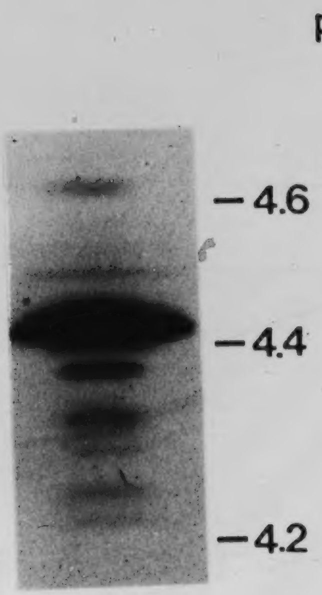

Fig. 3-8. Isoelectric focussing polyacrylamide gel electrophoresis of S-protein (40 μg protein).

three tryptophan residues per molecule. This is believed to be a reliable estimate since the method also indicated 16 tyrosine residues, which was in reasonable agreement with the 14 residues determined from the automated amino acid analysis.

Circular dichroism of S protein

The far-UV circular dichroism (CD) of S-protein is presented in Figure 3-9A. Calculations of the secondary structure based on the equations of Chen *et al.* (1972) indicated little or no α-helical structure (< 2%), 34% ß-structure, with the remainder of the polypeptide backbone being aperiodic in nature. S-protein was incubated with 1 mM EDTA (ethylenediamine tetraacetic acid) overnight at 4°C, but this treatment did not alter the secondary structure as detected by CD. The near-UV CD spectrum (Fig. 3.9B) showed negative dichroic troughs centered at 263 and 269.5 nm which were due to phenylalanine and another trough at 277 nm which was due to tyrosine (Strickland, 1972). As with the far UV-CD spectrum, there was no effect of EDTA on the near UV-CD spectrum indicating that the environments of the phenylalanine and tyrosine residues which contributed to the near-UV CD spectrum were not affected by this treatment. Treatment of S-protein with SDS (final concentration, 1% w/v) resulted in a 14% increase in α-helix and a slight increase in ß-structure (Fig. 3-9A) with a concurrent loss of aperiodic structure, which decreased from 65-70% to approximately 25% of the backbone. While the sum of the α-helical, ß-sheet and random coil components equalled unity for untreated (or EDTA treated) S-protein, the sum of the fractional coefficients only amounted to 0.81 for the SDS treated sample. However, only as little as a 10% negative error in the determination of the ellipticity at 225 nm can halve the proportion of random coil with only a 1-2% change in the proportion of α-helix and ß-sheet structure. Concomitant with the SDS binding was the loss of the 196 nm minimum seen in the CD spectrum of native S-protein. A minimum at 194 nm is characteristic of the unordered form of proteins (Chen *et al.*, 1972). This minimum was slightly red-shifted for the *A. vinelandii* S-protein and was closer to that observed for synthetic polypeptides. The loss of this minimum is further qualitative support for an enhancement of secondary structure on SDS binding.

The effect of various reagents on S-protein conformation was also investigated by electrophoresis in native polyacrylamide gels. S-protein (1 mg/ml) was treated overnight at room temperature with 1% SDS, sodium lauroyl sarcosine (Sarkosyl) or octyl phenoxyethanol (Triton X-100), 10mM ethyleneglycol-bis-(ß-aminoethylether) N,N'-tetraacetic acid (EGTA) or EDTA, (pH 8) and 10% w/v ß-mercaptoethanol. Samples were

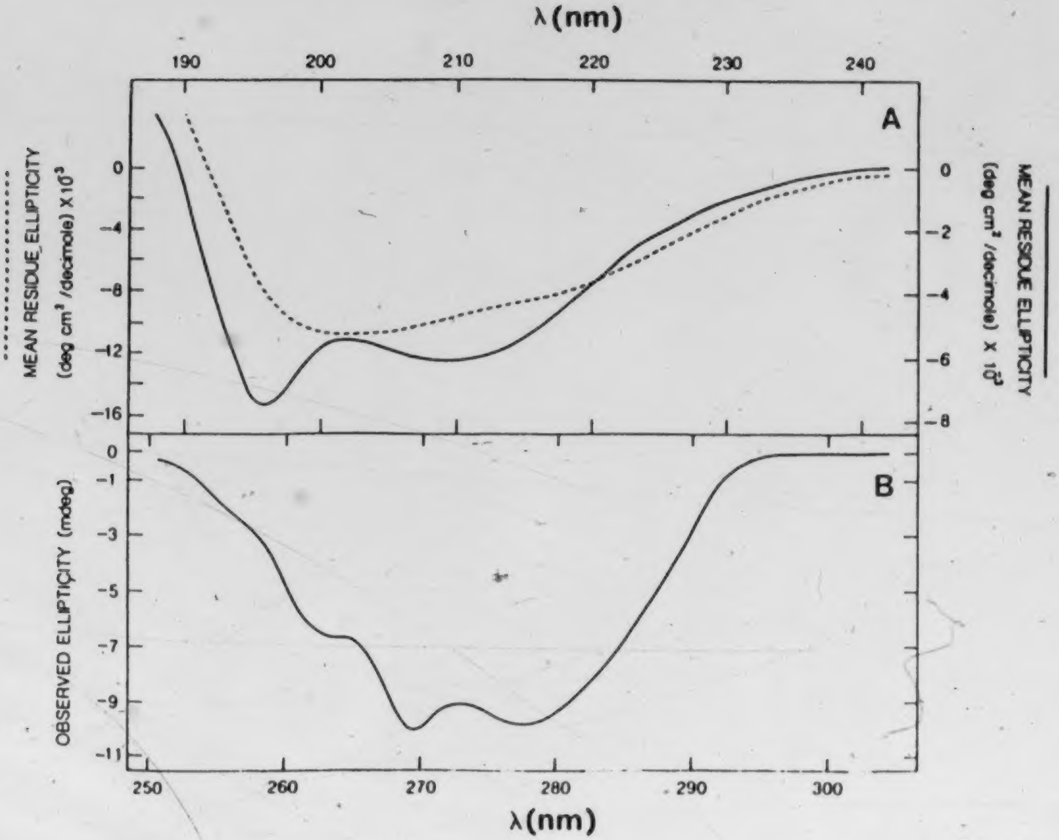

Fig. 3-9. Circular dichroism spectra of S-protein from *A. vinelandii* UW1 S-protein dissolved in 10 mM Tris/HCl, pH 7.5. (A) Far-UV CD spectrum, ———— , Native; ----- , 1% SDS. (B) Near UV-CD spectrum.

added to nondenaturing sample buffer containing 62.5 mM Tris/HCl, pH 6.8 and 10% glycerol and electrophoresed under nondenaturing conditions. The mobility of the protein was unaffected by all reagents except SDS (as expected) and Sarkosyl. SDS produced a smearing of the band while Sarkosyl produced some (even discrete) forms of S-protein with reduced mobility indicating a conformational change had been produced in the protein by this mildly anionic detergent (Fig. 3-6; lane 2).

Interaction of S-protein with divalent cations

The ability of divalent cations to produce conformational changes in purified S-protein was examined by circular dichroism. No change in either the near or far-UV CD spectrum was detected upon incubation of the protein with 1 mM $CaCl_2$ or 1 mM $MgCl_2$ which indicated that no conformational changes were produced by these species (at least those detectable by CD).

To test whether S-protein monomer could be reassembled into a multimeric form *in vitro* under the conditions prevailing in the reassembly assays, i.e., a protein concentration of 200 μg/ml, it was necessary to label S-protein with ^{35}S for detection purposes. Although the amino acid analysis indicated, contrary to the findings of Schenk and Earhart (1981), that significant amounts of cysteine were not present in the protein the presence of methionine allowed S-protein to be labelled with ^{35}S. However, when the gradient fractions were assayed for evidence of S-protein aggregation, the only radioactivity detected was in those fractions corresponding to S-protein monomer (Fig. 3-10B). This indicated both $MgCl_2$ or $CaCl_2$ failed to aggregate the monomer into higher molecular weight species.

The lack of any demonstrable effects of divalent cations on purified S-protein monomer was surprising considering that *in vitro* reassembly of S-protein onto the surface of distilled water washed cells was a divalent cation dependent event. It was subsequently found that purified S-protein monomer could not reassemble *in vitro* onto the surface of distilled water washed cells. S-protein in $BBPO_4$ buffer was incubated with distilled water washed cells in the presence of Ca^{2+} and Mg^{2+}, but no reattachment of the purified monomer to the cells was observed. The reason for the inability of purified S-protein to reassemble onto the cell surface *in vitro* was not immediately clear. S-protein freshly extracted from outer membrane fragments when mixed in excess with distilled water washed cells in the presence of Ca^{2+} or Mg^{2+} typically was found to cover the entire cell surface in a regular tetragonal pattern. When this freshly isolated S-protein was subjected to rate zonal centrifugation (Fig. 3-10A), two sedimenting zones were observed: one corresponding to S-protein monomer and a multimeric form which sedimented just behind jack bean urease trimer (molecular weight = 290,000; Dixon *et al.*, 1980). This suggested that the majority of the S-protein freshly prepared from outer membrane was present in a multimeric form and this was the species active in divalent cation mediated reassembly. It appeared during manipulations involved in the purification of S-protein, the multimeric species dissociated into monomers.

3.4 Discussion

The S-protein of *A. vinelandii* is similar to surface array proteins of many other eubacteria in its ability to self-assemble *in vitro* (Sleytr and Messner, 1983). Usually self-assembly is classified as either template or non-template dependent (Kist and Murray, 1984). The ability of the S-protein of *A. vinelandii* to self-assemble in the absence of an underlying cell wall template has not been investigated, mainly because the examination of the process really sheds no light on the *in vivo* process of assembly where a cell wall template is indisputably involved. Secondly, reassembly requirements in the absence of a cell wall may be quite different from those in the presence of a cell wall template (Thorne *et al.*, 1975). The demonstration of *in vitro* self-assembly of S-protein demonstrates that the protein released from purified outer membrane fragments retains at least the ability to attain a native configuration.

The level of reattachment of S-protein mediated by the divalent cations Ca^{2+}, Sr^{2+}, and Mg^{2+} only approached 60% because the amount of S-protein used was in excess of that required to saturate the cell surface. This amount of S-protein was used to ensure proper coverage of the cell for the companion freeze-etch study. Extraction of outer membrane fragments with distilled water at a concentration of 1 mg/ml outer membrane protein releases approximately 300 μg S-protein, the vast majority of which can subsequently be reattached to the vesicles upon addition of cations (Bingle *et al.*, 1984; Chapter 2). This observation served as the basis for the development of a reassembly assay for whole cells. Data from many experiments involving washing whole cells and determining the amounts of S-protein released, indicated an average of about 12-13 μg per OD_{620} unit; therefore, 300 μg S-protein represented about 26 OD_{620} units of cells. To maintain the same concentration of surface exposed per millilitre as found in reaction mixtures involving outer

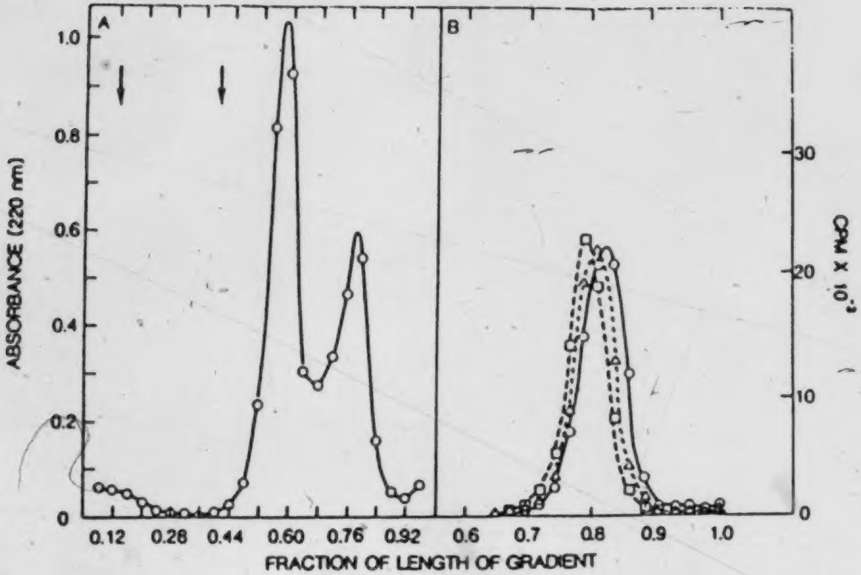

Fig. 3-10. Rate zonal sedimentation of S-protein. (A) Freshly isolated S-protein in 10 mM Tris/HCl, pH 7.5; (B) ^{35}S-labelled S-protein monomer preincubated in distilled water (○), 1 mM $CaCl_2$ (△), and 1mM $MgCl_2$ (□). Arrows left and right respectively indicate the sedimentation position of jack bean urease hexamer (molecular weight = 580,000) and trimer (molecular weight = 290,000). In order to detect the formation of multimers, ^{35}S-labelled S-protein monomer in distilled water was incubated with 1 mM $CaCl_2$ or 1 mM $MgCl_2$ at a concentration of 200 µg/ml for 2 h. A 200 µl aliquot was loaded on a glycerol gradient made in distilled water containing 1mM $CaCl_2$ or 1 mM $MgCl_2$ and centrifuged as described in Materials and Methods.

membrane fragments, 26 OD_{620} units of distilled water washed cells were resuspended in 1 ml of a 300 μg/ml solution of S-protein. However, this was quite a thick cell suspension to manipulate, so the concentration of exposed surface was decreased arbitrarily by one-half. In order to provide excess S-protein to the distilled water washed cells, the amount of S-protein was also increased by approximately one-half.

Divalent cations can mediate reassembly of the S-layer on whole cells at ionic strengths where monovalent cations are ineffective; this suggests a specific interaction between divalent cations, S-protein and the outer membrane. Cations may be involved in protein-protein interactions to form subunits, subunit-subunit interactions to form a tetragonal array, or a combination of these interactions. S-protein attached to whole cells in the presence of Mg^{2+}, Ca^{2+} and Sr^{2+} was organized into a regular array. In contrast, Be^{2+} promoted effective reattachment, but did not support regular array formation. Secondly, the reattachment curve for Be^{2+} was different from those obtained with the other divalent cations. Because of its small ionic radius and increased electronegativity, Be^{2+} has a greatly increased charge/radius ratio, relative to Ca^{2+}, Mg^{2+} and Sr^{2+}. This property causes beryllium bonding to possess appreciable covalent character with a shorter bond length than the ionic bonds characteristically formed by Ca^{2+}, Sr^{2+} and Mg^{2+} (Cotton and Wilkinson, 1980). Beryllium, therefore, may prevent the correct organization of S-protein into subunits or that of subunits into a regular array (or both), although it does attach unorganized S-protein to the cell surface. The *A. vinelandii* S-layer seems to be similar to that of *Acinetobacter* 199A in that a number of divalent cations will support reassembly of the S-layer into a regular tetragonal array (Thorne *et al.*, 1975).

Extraction of S-protein from outer membrane fragments is fast, producing reasonably concentrated solutions (1 mg/ml) and the protein spends minimal time free of the outer membrane. Such a preparation of S-protein is present in a highly purified state after a preliminary desalting step which appears to remove ribo- and deoxyribonucleotides. Most other S-proteins of gram-negative bacteria are released from the outer membrane with urea, guanidine/HCl or detergents (Koval and Murray, 1984) which can also release other outer membrane proteins and LPS (Phipps *et al.*, 1983; Thornley *et al.*, 1974), contaminants which must subsequently be removed. Distilled water extraction is a relatively benign treatment, highly specific for the *A. vinelandii* S-protein, with no appreciable release of any other outer membrane component.

S-protein exhibits anomalous behaviour on SDS-polyacrylamide gels, producing one major band and three minor bands when pretreated in SDS-sample buffer at 100°C. Some of these bands have been noted previously (Page and Doran, 1981) but not by Schenk and

Earhart (1981). These bands are due to conformational isomers of S-protein as shown by electrophoresis of samples treated with 2% SDS at different temperatures. Conformational isomers can be resolved by gel electrophoresis if the half time of isomerization (unfolding) is on the order of, or significantly greater than, the time of electrophoresis (Goldenberg and Creighton, 1984). Usually only native and completely unfolded states predominate because partially folded states are unstable. However, intermediates may be visible if the protein contains a number of independently unfolding domains. For the *A. vinelandii* S-protein there appears to be a number of relatively stable intermediates. The pattern of bands between the 30,000 and 55,000 forms depicted in Figure 3-5 is exactly what is predicted by theory for irreversible isomerization of the folded to unfolded states (Goldenberg and Creighton, 1984), although the interpretation is complicated somewhat by the presence of discrete conformational isomers. Since discrete conformational isomers are visible, clearly the major unfolding of S-protein occurs during the pretreatment in 2% SDS sample buffer. Little further conversion occurs during electrophoresis in gels containing 0.1% SDS although the smearing in the 20°C and 30°C lanes may indicate some isomerization during electrophoresis. The discrete conformational isomers seen between the 30K and 55K forms show the greatest net production at 40°C as does the 16K band indicating that this band is also a conformational isomer. However, the combination of SDS binding and hydrodynamic volume which produces such a high mobility is difficult to visualize. In contrast to this study, Schenk *et al.* (1977) reported that the migration of S-protein in SDS gels was not affected by temperature of solubilization. However, only temperatures between 37°C and 100°C were tested and samples heated in excess of 40°C can produce only traces of the 45K, 30K, and 16K bands when small amounts of protein are employed. The S-protein of *C. crescentus* also behaves anomalously when electrophoresed in SDS-gels. When this protein is isolated using chaotropic agents, it will only enter SDS gels if treated in SDS-sample buffer at less than 40°C (Smit and Agabian, 1984). It has been suggested that multiple low molecular weight bands on SDS-PAGE result from proteolytic degradation of S-layer proteins during isolation (Koval and Murray, 1984). The present study with the *A. vinelandii* S-protein indicates another explanation for multiple bands on SDS-PAGE, that is, conformational isomers. Whether this is unique to the *A. vinelandii* surface array protein is unknown.

S-protein produces a single band on nondenaturing gels contrary to the findings of Schenk and Earhart (1981) who reported that S-protein would not enter such gels. A single major isoelectric form (pI 4.4) is seen by IEF-PAGE but several minor bands are also evident. Multiple isoelectric forms have been noted for other surface array proteins and result from differences in deamidation which occurs *in vivo* or during purification (Phipps *et al.*, 1983). Page and Doran (1981) reported a pI of 5.1 for S-protein isolated from whole cells, but the pH gradient employed may have led to problems in accurately determining the pI value at the extreme acid end of the gradient.

The amino acid analysis of the *A. vinelandii* S-protein is similar to that published for other surface array proteins (Sleytr and Messner, 1983) but somewhat different from that reported by Schenk and Earhart (1981). Their analysis indicated significant quantities of cysteine and glutamic acid, the latter amino acid constituting 20% of the protein. It is extremely unusual for surface array proteins to contain cysteine although exceptions apparently exist (Baumeister *et al.,* 1982). Further evidence for the lack of cysteine is that the mobility of S-protein in SDS and native gels is unaffected by pretreatment with ß-mercaptoethanol, a finding also reported by Schenk *et al.* (1977). The differences in the amino acid analysis of S-protein between this study and the study of Schenk and Earhart (1981) could probably be attributed to the source of S-protein and its preparation. Although not reported in Schenk and Earhart (1981) their preparation contained 80 mg carbohydrate per mg protein (Schenk, 1978) yet immunological tests revealed no capsule or LPS in the sample. Such a large amount of carbohydrate may have complicated the amino acid analysis. The theoretical molecular weight for S-protein calculated from the amino acid analysis is 60,218. Schenk and Earhart (1981) calculated a value of at least 65,460 and the actual molecular weight was thought to be higher because of the presence of some unidentified amino acid peaks. However, no unassignable amino acid peaks were found in the samples analyzed for this study. These authors also failed to subtract the peptide bond water contribution to the molecular weight of S-protein. Taking this factor into account would bring their theoretical molecular weight to about 56,000 not 65,000.

The secondary structure of S-protein as estimated by circular dichroism shows negligible α-helix, about 35% ß-structure and a high level of aperiodic structure. Nearly identical results were obtained by Baumeister *et al.* (1982) for the S-protein of *D. radiodurans* using infrared spectroscopy to estimate secondary structure. These results are also somewhat similar to those of Phipps *et al.* (1983) for the S- protein of *A. salmonicida* which was estimated to have 14% α-helix and 19-28% ß-structure, with the majority of the backbone existing in an apparent random coil configuration. Sleytr and Messner (1983) calculated, based on the method of Krigbaum and Knutton (1973) which attempts to predict secondary structure from amino acid composition, that all S-proteins should possess 40% α-helix and 20% ß-sheet structure. Clearly this prediction is not borne out by the data that have accumulated so far. A further examination of the method of

Krigbaum and Knutton (1973) using the data of Rosenbusch (1974) showed that it also did not predict the predominantly ß-sheet type of secondary structure found in the *E. coli* matrix protein (Rosenbusch, 1974). It is not surprising that prediction of secondary structure from amino acid composition alone is a dubious proposition. Recently, the secondary structure of the S-protein from the archaebacterium *Halobacterium halobium* was published (Hecht *et al.*, 1986). The CD spectrum of this protein (in the presence of 3 M NaCl) also indicates a high level of ß-structure suggesting this characteristic may be a general one. For an unknown reason Hecht *et al.* (1986) reported that the CD spectrum of the S-protein of *H. halobium* was typical of a "helical" protein. However, examination of the CD spectrum clearly indicates ß-structure.

Treatment of S-protein overnight with EDTA had no effect on the near or far-UV CD spectra indicating that divalent cations may not influence the secondary and tertiary structure of the protein, but it is also possible that any divalent cations important in thisregard were lost during purification. SDS significantly enhanced the proportion of α-helix in S-protein apparently at the expense of aperiodic structure. An enhancement of α-helical structure by SDS was noted for the S-protein of *A. salmonicida* (Phipps *et al.*, 1983). As pointed out by these workers and others (Tokunaga *et al.*, 1979) such a characteristic is not uncommon for proteins which are rich in ß-structure in their native states. Schenk (1978) reported that Sarkosyl dissociated S-protein from the cell wall. This was also observed by Page and Huyer (1984) who speculated this detergent bound divalent cations important to the attachment of S-protein to the cell wall but this explanation is probably unlikely considering the negligible effect of EDTA on the conformation of S-protein; the action of Sarkosyl is more likely similar to SDS. The nonionic detergent Triton X-100 produced no effect on the mobility of S-protein which suggests that this detergent may be useful in an alternative and less laborious method of preparing outer membranes with attached S-protein than the use of sucrose gradients. Exploratory experiments showed that 1% Triton X-100 solubilized the cytoplasmic membrane of *A. vinelandii*, allowing the recovery of outer membranes with attached S-protein. However, a visible pellet of PHB was also present in outer membrane preparations which would have to be removed via a density gradient approach. This drawback, as well as the need to remove residual Triton X-100, argued in favour of retaining the use of sucrose density gradients for the recovery of outer membranes.

While the reassembly of S-protein onto the surface of distilled water washed cells is a divalent cation dependent event, purified S-protein monomer appears indifferent to the presence of these species. Circular dichroism is a well established method for detecting conformational changes in proteins induced by cation binding (Klevit, 1983). Nevertheless, no conformational changes are seen in S-protein by this method. A divalent cation concentration of 1 mM was chosen because this level supports both *in vivo* assembly and *in vitro* reassembly of the S-layer. This does not rule out the possibility that higher concentrations of cations may induce conformational changes in S-protein but the biological significance of such changes would be difficult to interpret. Divalent cations do not aggregate S-protein monomer into multimers in the presence, or absence, of a cell wall template. Assuming the morphological subunit of the array to be a tetramer (no real evidence for this exists) a change in the quaternary structure of S-protein is necessary for the formation of the morphological subunit. These data, in conjunction with the finding that S-protein active in reassembly is already preassembled into a multimeric species, suggests that S-protein possesses a limited capacity for *in vitro* self-assembly. Although quite gentle methods were used during purification, S-protein multimer dissociated into monomers. Thus, an investigation is needed of the properties of the multimeric form of S-protein and its stability once dissociated from the cell wall.

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4. Structure of the *Azotobacter vinelandii* surface layer

4.1 Introduction

Surface layers of regularly arranged protein subunits are distributed in most and probably all of the ten major eubacterial lines of descent defined by 16S rRNA cataloguing (Sleytr and Messner, 1983; Woese *et al.*, 1985). Because the majority of eubacterial S-layers are of hexagonal, rather than tetragonal organization, studies detailing the fine structure of hexagonal S-layers have dominated the literature. These studies, employing computer image enhancement of electron micrographs of negatively stained S-layer preparations, have led to the recognition of common structural features of hexagonal S-layers at a gross level (Baumeister *et al.*, 1986). Until 1986, only the tetragonal S-layers of the spore-forming gram-positive bacteria had been resolved to a sufficient level for structural comparisons, and a general model of their organization was proposed (Burley and Murray, 1983). However, recently, image processing techniques were applied to the tetragonal S-layer of the gram-negative bacterium *Aeromonas salmonicida* (Stewart *et al.*, 1986). This study presents the structure of the second tetragonal S-layer from a gram-negative bacterium, *Azotobacter vinelandii*.

A unique characteristic of the *A. vinelandii* surface array is that it can be removed from whole cells or isolated outer membrane fragments by simple distilled water extraction (Schenk *et al.*, 1977). Distilled water extraction of the *A. vinelandii* S-layer releases primarily a multimeric form of S-protein possessing a molecular weight in the neighbourhood of 200,000 (Bingle *et al.*, 1986); this figure is tantalizingly close to that expected for the morphological subunit of the array, assuming it is a tetramer. The ability to isolate intact morphological subunits could allow complementation of projection views of the S-layer, using image processing techniques, with biochemical data on the building block of the array, information which is rarely, but sometimes (Baumeister *et al.*, 1982) included in such ultrastructural studies. However, multimeric S-protein exhibits low intrinsic stability once released from the outer membrane, dissociating into monomers during incubation in a number of buffers. Thus, in order to conduct experiments with multimeric S-protein, it is necessary to determine the reason(s) for its lability once free of

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the outer membrane, and to develop a way to stabilize it in solution. This chapter presents (1) a two dimensional average of the *A. vinelandii* S-layer, based on conventional Fourier based computer-assisted image processing techniques, (2) an investigation of the stabilization of multimeric S-protein with divalent cations and (3) and evaluation of its molecular weight and secondary structure.

4.2 Materials and Methods

Bacterial strains and growth conditions

A. vinelandii UW1 was grown in Burk minimal medium as previously described (Bingle *et al.*, 1984; Chapter 2). ^{35}S-labelling of whole cells was accomplished by including $^{35}SO_4^{2-}$ (as H_2SO_4) in the growth medium at a specific concentration of 0.5 mCi/ 100 ml (Bingle *et al.*, 1986; Chapter 3). $BBPO_4$ and OFeBB have been defined previously (Bingle *et al.*, 1986; Chpater 3).

Electron microscopy and image processing

Cells were washed once in Burk buffer, and resuspended for sonication for 1 min in an ice bath. Unbroken cells and larger fragments were separated by centrifuging at 3,000 g and small wall and envelope fragments were separated from the supernatant at 12,000 g for 15 min; the latter pellet was resuspended in Burk buffer. Carbon-Formvar coated grids were floated on droplets of the suspension and then transferred to droplets of saturated ammonium molybdate (ca. 4%) in Burk buffer (pH not adjusted, ca. pH 5.5-6). After 1 min, the grids were removed and excess fluid was aspirated against the edge of bibulous paper. Grids were then examined in a Philips electron microscope (EM-300) at 60 KV and micrographs taken on a fine grain positive film.

A negative at 14,000X and an area on that film were selected for minimum astigmatism, appropriate focus and maximum information using an optical diffractometer (Burley *et al.*, 1983). An area of 26 mm^2 on the negative was digitized on a flat bed microdensitometer originally built by Dr. D.J. De Rosier, Brandeis University, using a step size of 20 μm to generate an array of 256 x 256 pixels. A Fourier transform was developed from the digitized area using a program written at the University of Western Ontario for a PDP 11-44 mini-computer (Burley *et al.*, 1983). A least squares fitting routine was used to determine the reciprocal lattice vectors and then the amplitudes and phases of the reciprocal lattice points were abstracted from the Fourier transform. A phase refinement program was used to determine the positions of the symmetry axes. The symmetry of this image requires that the phases be either 0 or 180 degrees, and the phase residual from these values, weighted with the amplitudes was 30 degrees using the third order reflections. The image reconstructed from the filtered Fourier transform was displayed on a monitor and this display was photographed to record the result.

Isolation of S-protein and reassembly assays

S-protein was extracted from outer membrane fragments as outlined by Bingle *et al.* (1986; Chapter 3). Briefly, outer membrane fragments were resuspended in distilled water and agitated by vigorous vortex mixing for one minute followed by removal of the outer membrane fragments by centrifugation. Concentration and desalting of S-protein solutions was done by ultrafiltration and gel filtration, respectively, as previously described (Bingle *et al.*, 1986; Chapter 3). Fresh S-protein was prepared for every experiment and was used within 2 h of being released from the outer membrane. Reassembly assays were conducted as described by Bingle *et al.* (1986; Chapter 3).

Separation of monomeric and multimeric S-protein

Multimeric S-protein was separated from the monomeric species on a preparative scale by Sephadex G-100 Superfine gel chromatography. A Pharmacia K 26/45 column was packed and the gel was equilibrated with 10 mM Tris/HCl, pH 7.5, or $BBPO_4$ buffer, pH 7.2, at 4°C and eluted with the same buffer at a flow rate of 10 ml/hr collecting 2.8 ml fractions.

Multimeric and monomeric S-protein were separated on an analytical scale by rate zonal centrifugation in linear glycerol gradients (6-30% v/v glycerol in 50 mM Tris/HCl, pH 7.5) as previously outlined (Bingle *et al* ., 1986). The gradients were centrifuged at 200,000 g (40,000 rpm; Beckman SW 40Ti rotor) for 21.5 h at 4°C prior to fractionation.

Molecular weight of S-protein multimer

The molecular weight of S-protein multimer was estimated by gel filtration. Sepharose 6B was packed in a Pharmacia K 26/100 column (bed volume, 522 ml) under 30 cm pressure using 50 mM Tris/HCl, pH 7.5, containing 1.5 mM $CaCl_2$ and 1.5 mM $MgCl_2$ as the elution buffer. The bed was washed with two column volumes of the same buffer at 4°C and freshly isolated ^{35}S-labelled S-protein was applied to the column and eluted at a flow rate of 11.2 ml/hr. The column was precalibrated with the following protein molecular weight standards (Sigma Chemical Co., St. Louis, Mo.): thyroglobulin (669,000), apoferrtin (443,000), amylase (200,000), alcohol deydrogenase (150,000) and bovine serum albumin (66,000). The best fit linear relationship between elution position and log molecular weight was determined by least squares analysis.

Circular dichroism (CD) of S-protein multimer

The CD spectrum of S-protein multimer in the presence and absence of 3 mM $CaCl_2$ was determined using a Cary 60 spectropolarimeter as described by Oikawa *et al.* (1968). The percent α-helix , ß-sheet and random coil configurations were calculated based onthe equations of Chen *et al.* (1972) as described by Bingle *et al.* (1986; Chapter 3).

Stability of S-protein multimer

The effect of buffer type, divalent cations and temperature on the stability of S-protein multimer was examined by rate zonal centrifugation. S-protein released from the outer membrane by distilled water extraction was concentrated by ultrafiltration, filter sterilized (0.22 µm pore size) and aliquots were placed in sterile 1.5 ml Eppendorf tubes followed by the appropriate addition of buffer and/or divalent cation species from concentrated stock solutions to give the desired final concentrations. After 3 days of incubation, 200 µl aliquots were removed and analyzed by rate zonal centrifugation on glycerol gradients.

After centrifugation, the gradients were fractionated into approximately 0.5 ml fractions using an automatic fraction collector. Fractions containing ^{35}S-labelled S-protein were collected in 1.5 ml Eppendorf tubes. These tubes were placed in scintillation vials, covered with ACS scintillation fluid (Amersham Corp. Oakville, Ontario) and mixed well. Fractions containing unlabelled S-protein were analyzed by determining their absorbance at 220 nm (A_{220}).

3.3 Results

Electron microscopic structure of the S-layer

Preparations of wall fragments generated from sonicated cells showed a number of sheets of tetragonal array with a lattice constant of 12-13 nm when negatively stained with ammonium molybdate dissolved to saturation in Burk buffer. These sheets included some that appeared not to be backed by a sheet of outer membrane and these were chosen for high resolution microscopy. Unfortunately even these choice sheets (Fig. 4-1A) showed an irregular distribution of patches which were probably remnants of the outer membrane.

The Fourier transform derived from the digitized image of a selected area (Fig. 4-1B) showed observable reflections to the third order. A detailed analysis of the amplitudes and phases showed that the symmetry of the image was p4. The phases were set to 0 or 180 degrees as appropriate and internally averaged for p4 symmetry. These were then used to refine the final filtered image (Fig. 4-1C). The direct lattice vectors for the unit cell a=b=18.4 nm, with a repeat frequency for the centres of mass in each unit cell of close to 13 nm. The resolution determined on this image was 4 nm which is lower than optimal for image processing. This was probably due in part to the additional electron scattering provided by the outer membrane patches.

The filtered image (Fig. 4-1C) gave the overall impression of an array consisting of p4 units alternating in apparent density, and the lesser of these rotated clockwise about 27 degrees. However, closer inspection shows delicate linkers from each of the four elements in the center of mass projecting left handedly to meet with similar linkers from the three other centers of mass in that quarter. Thus the unit cell (outlined in Fig. 4-1C) appeared to consist , probably, of four macromolecules, each with a delicate extension forming the linkers of the array. The centers of mass showed a partial filling with negative stain, i.e., not a hole but rather a depression. The boundaries of the spaces (or holes) between the units were taken to be uncertain considering the relatively low resolution of the image.

Isolation of multimeric S-protein on a preparative scale

Previous studies indicated that S-protein freshly extracted from outer membrane fragments was present primarily as a multimeric species (2:1 ratio of multimer to monomer) of molecular weight in the neighbourhood of 200,000. An attempt was made to isolate a homogeneous preparation of this species on a preparative scale for further study.

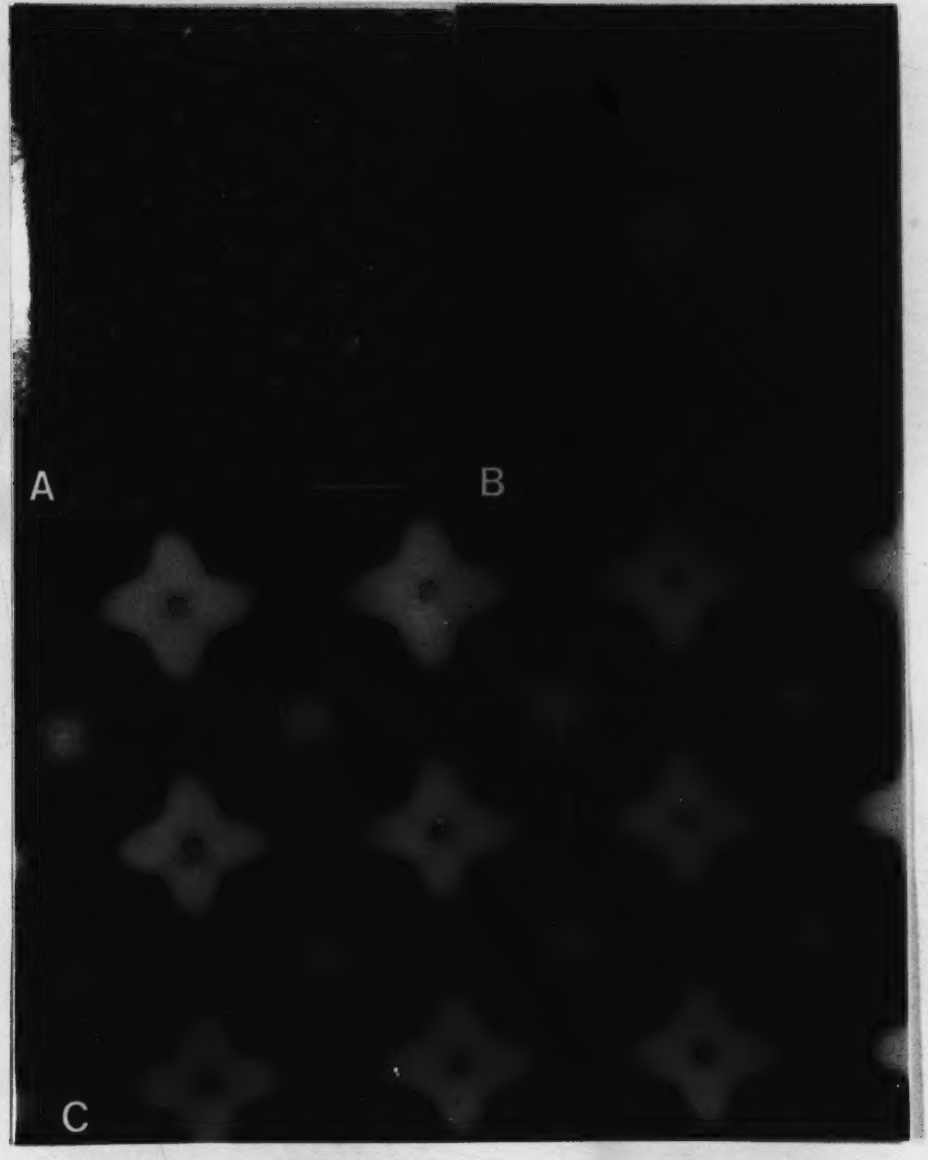

Fig. 4-1. A) Electron micrograph of the S-layer of *A.vinelandii* negatively stained with ammonium molybdate. The tetragonal array shows a repeat frequency of ca. 12.5 nm. The irregular patches are probably remains of the underlying outer membrane. A central area was digitized for image analysis. Bar, 100 nm. (B) Fourier transform derived from a digitized image of (A). (C) Reconstructed image from a filtered Fourier transform showing the linkage pattern for the units forming the tetragonal array. One unit cell is outlined in white.

A preparation of S-protein freshly extracted from outer membrane fragments was concentrated by ultrafiltration and chromatographed on Sephadex G-100 Superfine as described in Materials and Methods. All manipulations were performed at 4°C, as quickly as possible and S-protein solutions were not subjected to any freeze-thaw cycles at any stage. The molecular weight distribution of freshly isolated S-protein determined by gel filtration was the same as previous experiments (Bingle *et al.*, 1986; Chapter 3) involving rate zonal centrifugation (Chapter 3), i.e., about two-thirds of the protein eluted at the void volume and was expected to be S-protein multimer while one-third eluted at the same position as bovine serum albumin (BSA) and was expected to be S-protein monomer (Fig. 4-2). S-protein in both the void volume and BSA fractions was tested for the ability to reassemble into a tetragonal array on the surface of distilled water washed cells after exchange of the Tris buffer for $BBPO_4$ buffer. However, neither S-protein from the void volume fractions or those in which BSA eluted, participated in divalent cation-mediated *in vitro* reassembly. This was not due to the chromatography *per se* because a subsample of protein held at 4°C over the same time period also lost all reassembly competence.

As surmised from previous work, storage of S-protein produced a shift in its molecular weight distribution with a loss of the multimer peak and an intensification of the monomer peak (Fig. 4-3). While the fresh preparation (approximately 85% multimer) was capable of *in vitro* reassembly the stored preparation (80% monomer) was not. For the experiment depicted in Figure 4-3, $BBPO_4$ buffer was substituted for Tris buffer during storage and chromatography which obviated the need for further manipulation of S-protein prior to performing reassembly assays.

Stabilization of S-protein multimer

Although the concentration of both the Tris and $BBPO_4$ buffers used in the storage experiments was low, both buffers could be expected to compete with, or sequester, respectively, divalent cations which may have been important to the stability of the multimer. However, dissociation of the multimer into monomers was not prevented by storage at 4°C in 10 or 50 mM K^+-HEPES, pH 7.5 , a buffer which does not bind cations (Gueffroy, 1981). Since low temperature may be expected to weaken hydrophobic interactions (Cantor and Schimmel, 1980) which may have been important to the stability of S-protein multimer, this factor was also investigated. S-protein multimer was incubated in 50 mM K^+-HEPES, pH 7.5, buffer at 4, 10, 15, 20, 25 and 30°C for 3 days after

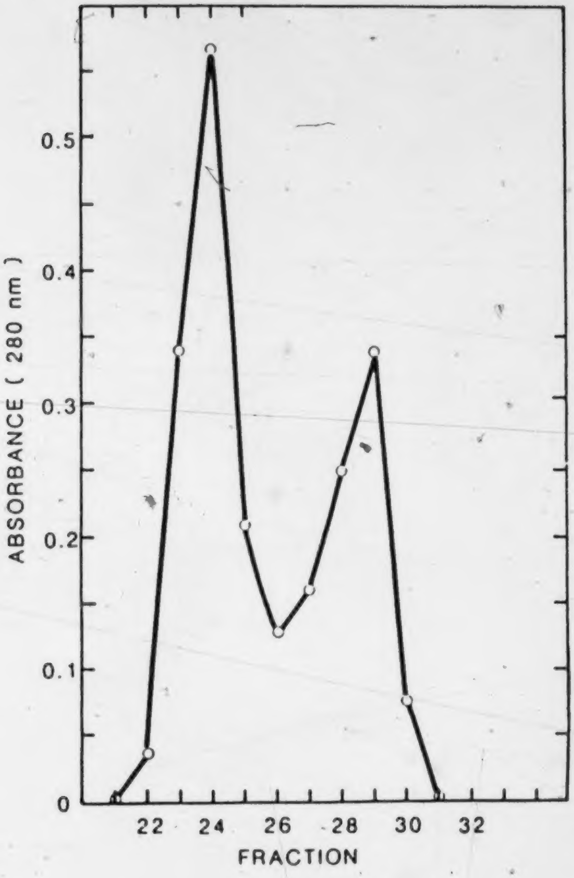

Fig. 4-2. Sephadex G-100 Superfine gel chromatography of S-protein freshly isolated from the outer membrane by distilled water extraction. The column was equilibrated with 10 mM Tris/HCl, pH 7.5 and S-protein was eluted with the same buffer at 4°C at 10 ml/h collecting 2.8 ml fractions. Blue dextran and BSA (molecular weight = 66,000) eluted in fractions 24 and 29 respectively.

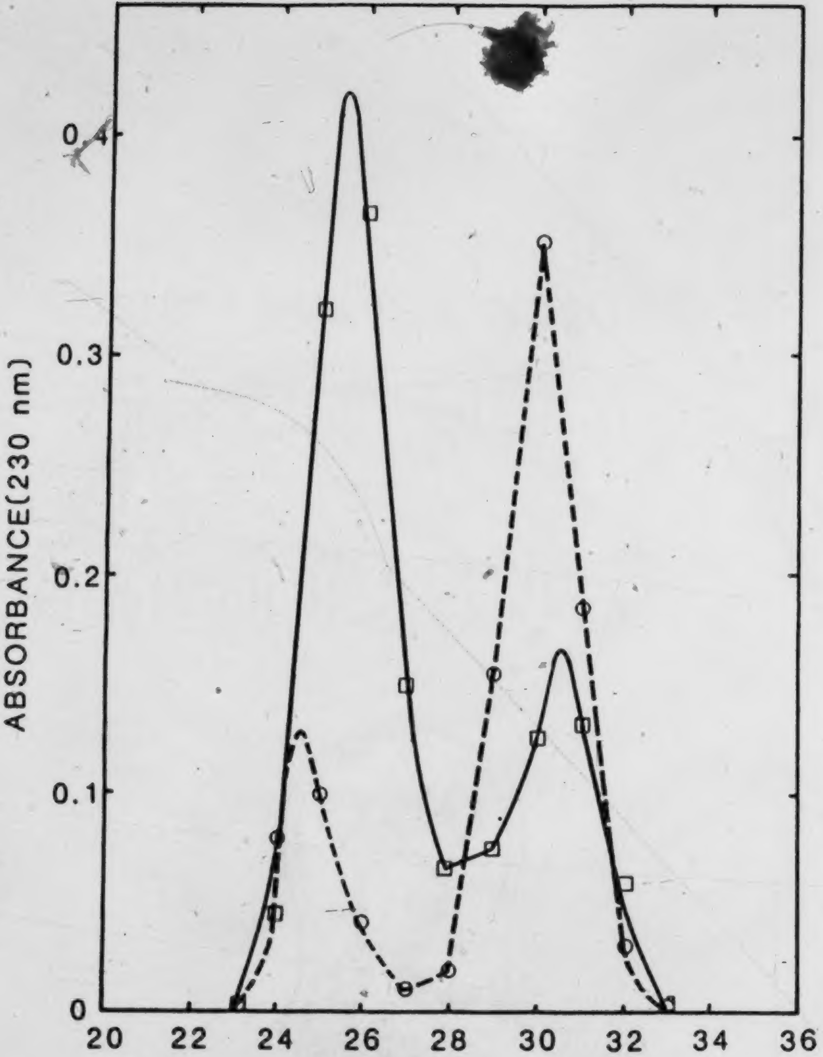

Fig. 4-3. Effect of storage in potassium phosphate buffer ($BBPO_4$) on the quaternary structure of S-protein determined by gel filtration on Sephadex G-100 Superfine. Fresh sample (□), S-protein stored for 3 days at 4°C (○). Blue dextran and BSA eluted in fractions 26 and 30 respectively.

which the amount of multimer and monomer in the S-protein solution was estimated by rate zonal centrifugation. However, the multimer experienced the same instability, dissociating into monomers, regardless of the temperature of incubation. Only when stored at 4°C was it possible to detect a trace amount of S-protein in the multimer fraction.

The effect of the divalent cations Ca^{2+} and Mg^{2+} was examined in a similar manner and to improve the quantitative nature of the experiment, S-protein was labelled with ^{35}S. S-protein multimer was incubated at a concentration of 4 mg/ml in 50 mM Tris/HCl, pH 7.5, with 1-10 mM Ca^{2+} or Mg^{2+}. After 3 days of storage at 4°C the amount of multimeric and monomeric ^{35}S-labelled S-protein was determined. Both Ca^{2+} and Mg^{2+} prevented dissociation of the multimer into monomers at concentrations of between 2-5 mM (Fig.4-4A and B). However, both species also caused extensive aggregation of S-protein. Preliminary experiments (Fig. 4-5) which involved subjecting S-protein to rate zonal centrifugation immediately after addition of divalent cations, indicated that the aggregates were formed from S-protein multimer only and not the monomeric form. This confirmed with fresh preparations of the monomer what had been found earlier with purified S-protein monomer (Bingle *et al.*, 1986; Chapter 3).

Because the monomeric form was not aggregated by divalent cations and the multimer appeared to be stabilized by the same species, the amount of S-protein monomer produced upon initial extraction of the outer membrane could be determined by subjecting a fresh sample of S-protein to rate zonal centrifugation in the presence of divalent cations. This was done prior to conducting the storage experiment depicted in Figure 4-4 and it was found that approximately 25% of the S-protein initially extracted from the outer membrane was present in the monomeric form. Thus, the monomer curves in Figure 4-4A and B approached an asymptote at a monomer level of 25%, i.e., the monomer level was never reduced to zero because a certain amount was produced upon initial extraction of the outer membrane and this material did not reassociate into multimers nor did it aggregate. Because the appearance of free monomer paralleled aggregation, the possibility was investigated that physical entrapment of the monomer was occurring rather than a true enhancement of multimer stability by divalent cations. The previous experiment was repeated at a divalent cation concentration of 5 mM using S-protein solutions of progressively lower concentration. As expected, dilution of S-protein produced a large decrease in aggregate formation concomitant with an increase in the proportion of both multimeric and monomeric S-protein (Fig. 4-4 C and D). The increase in the proportion of monomer indicated that the stability of S-protein multimer was somewhat dependent on protein concentration. However, the increase in monomer levels over the protein

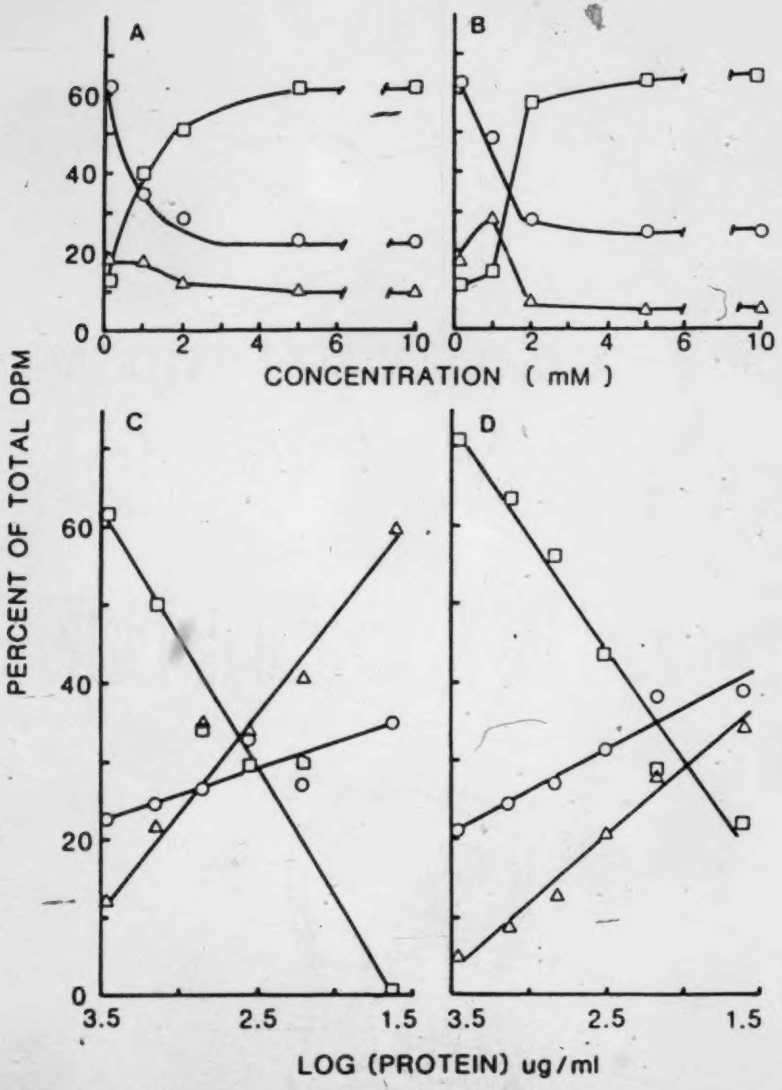

Fig. 4-4. Effect of divalent cations and protein concentration on the stability of ^{35}S-labelled S-protein multimer. Freshly isolated S-protein (4 mg/ml) was incubated with (A) Ca^{2+} and (B) Mg^{2+} for 3 days and subsequently analyzed by rate zonal centrifugation. Freshly isolated ^{35}S-labelled S-protein multimer was incubated with 5 mM (C) Ca^{2+} or (D) Mg^{2+} at various protein concentrations for 3 days before determination of the proportion of monomer (○), multimer (△) and aggregates (□) by rate zonal centrifugation.

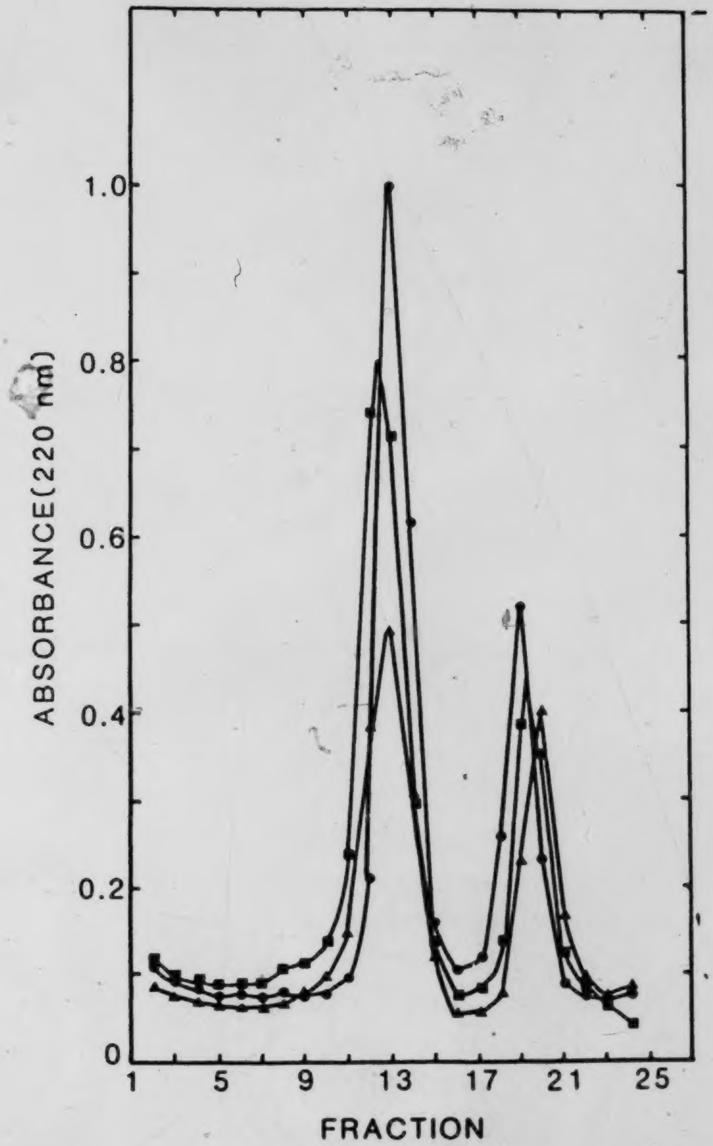

Fig. 4-5. Effect of divalent cation concentration on the aggregation of S-protein monomer and multimer. Freshly isolated S-protein in 50 mM Tris/HCl, pH 7.5 was mixed with equimolar concentrations of Ca^{2+} and Mg^{2+} to give the following final concentrations: 2 mM (●), 5 mM (■) and 10 mM (▲) and immediately subjected to rate zonal centrifugation in glycerol gradients containing the same concentrations of Ca^{2+} and Mg^{2+}.

concentration range tested was less than two-fold. In contrast, multimer levels increased 6-7 fold with reduction in S-protein concentration, indicating that the decrease in monomer levels with increasing cation concentration (Fig. 4-4A and B) was not due to nonspecific physical entrapment but was a result of a true enhancement of multimer stability. Both Ca^{2+} and Mg^{2+} produced the same result but differed quantitatively in the resulting proportions of aggregates, multimer and monomer (Fig. 4-4C and D).

Molecular weight of S-protein multimer

^{35}S-labelled S-protein freshly extracted from the outer membrane was chromatographed on Sepharose 6B in the presence of 1.5 mM $CaCl_2$ and 1.5 mM $MgCl_2$ as described in Materials and Methods. The protein was labelled because it was expected that the divalent cations used in the elution buffer would cause extensive aggregation of S-protein multimer leaving only small quantities of residual free multimer which would have been difficult to detect spectrophotometrically. However, the majority of the protein eluted as the free multimer with only minor amounts of aggregated material eluting at the void volume (Fig. 4-6). It was not possible to include the standard 100 mM NaCl in the elution buffer since the high salt concentration produced unacceptable aggregation of the multimer although it had no effect on the monomeric species. The low ionic strength conditions during gel filtration may have produced more scatter in the molecular weight calibration curve (Fig. 4-6 : inset) than normally encountered but this did not appear serious. When the K_{av} values for the molecular weight markers were calculated and compared to the literature (Pharmacia Fine Chemicals, 1984), all except apoferritin eluted within 5-6% of their expected position. Apoferritin behaved anomalously and was retarded by the column much more than expected, indicating a probable interaction between this protein and the gel matrix under the low ionic strength conditions employed. Therefore, this standard was discarded when calculating the best fit linear relationship between log molecular weight and elution position for the calibration standards. Gel filtration chromatography indicated that S-protein multimer possessed a molecular weight of 255,000 suggesting that it was a tetramer of four identical subunits of 60,000 molecular weight each.

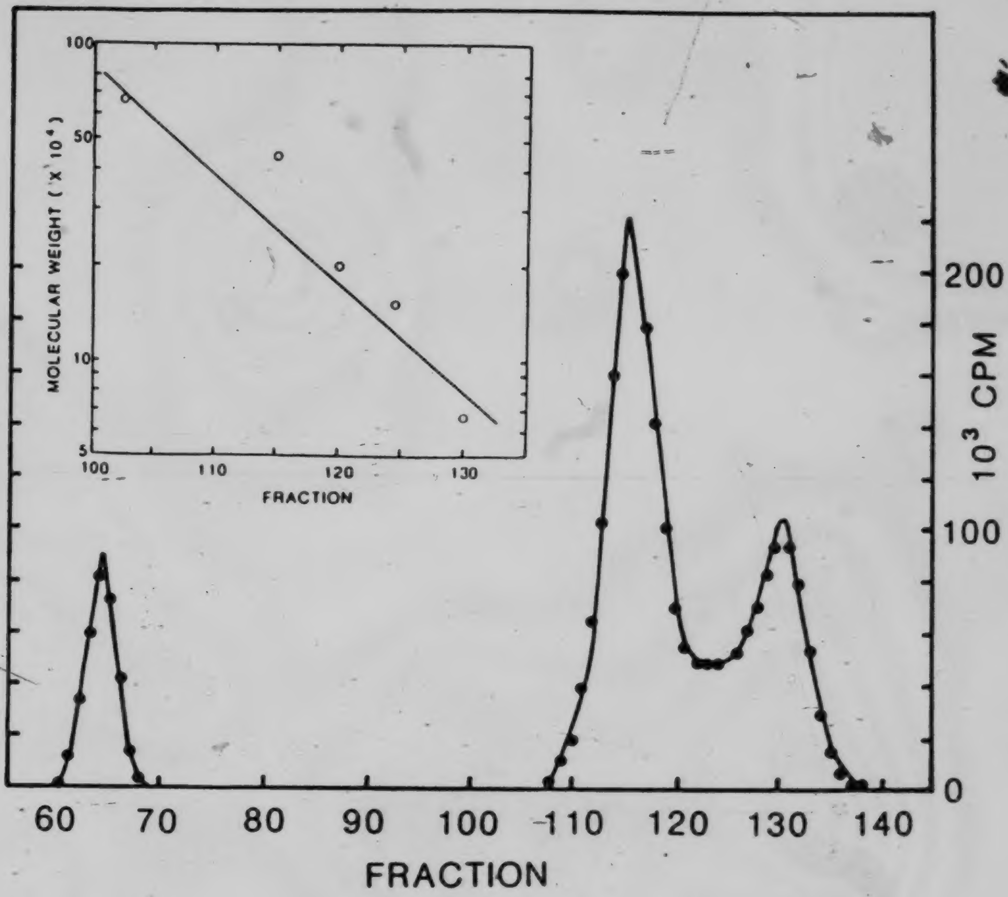

Fig. 4-6. Molecular weight determination of S-protein multimer by Sepharose 6B gel filtration chromatography. ^{35}S-labelled S-protein multimer was chromatographed in the presence of Ca^{2+} and Mg^{2+} as described in Materials and Methods. Inset: Molecular weight calibration curve; ordinate molecular weight X 10^4.

Secondary structure of S-protein multimer

A rare preparation of S-protein freshly extracted from the outer membrane which was composed of at least 95% multimer (Fig. 4-7: inset) allowed an estimation of the secondary structure of the multimeric form of S-protein using circular dichroism measurements. These measurements indicated that S-protein multimer possessed approximately 4% α-helix and 35% ß-sheet structure with the remainder in an apparent random coil configuration (Fig. 4-7) . Addition of $CaCl_2$ (3 mM) had no appreciable effect on the CD spectrum above 213 nm. Since the values of mean residue ellipticity used to estimate the percent α-helix, ß-sheet and apparent random coil configurations in conjunction with the equations of Chen *et al.* (1972) were those corresponding to wavelengths of 213 nm or greater, this analysis did not indicate any quantitative change in the secondary structure. Below 213 nm, differences in the CD spectra were evident, and the lack of correspondence between the two spectra intensified with decreasing wavelength. These apparent differences in mean residue ellipticity at shorter wavelengths were attributed to the increased noise levels encountered in this region of the spectrum, rather than any gross change in the secondary structure produced in response to Ca^{2+}.

4.4 Discussion

The details of the construction of the S-layer of *A. vinelandii* show a startling similarity in principle to the "Type II" array on *A. salmonicida* demonstrated by Stewart *et al.* (1986). In this case, the molecular weight of the protein is slightly larger (60K versus 50K), the alternating lower density elements are rotated somewhat more (27 degrees versus 23 degrees), the centres of mass are more cruciform, the centre linkage is relatively less dense, and the apparent lattice constant is 50% larger. As in *A. salmonicida* the true unit cell (see Fig. 4-1C) is defined by the centres of the linkage (" units of the other sort", Stewart *et al.*, 1986) surrounding the centre of mass.

This array represents an interesting class of array for gram-negative bacteria, but in principle, not unlike the linkage form in the generalized plan envisioned by Burley and Murray (1983) for the tetragonal arrays of some *Bacillus* species. However, unlike the spore-forming gram-positive bacteria whose arrays of morphological subunits possess connectivity at both the two-fold and one of the two four-fold rotational axes, the array of *A. vinelandii* does not possess any connectivity at the two-fold rotational axis. With respect to the general plan describing S-layer organization (Baumeister *et al.*, 1986),

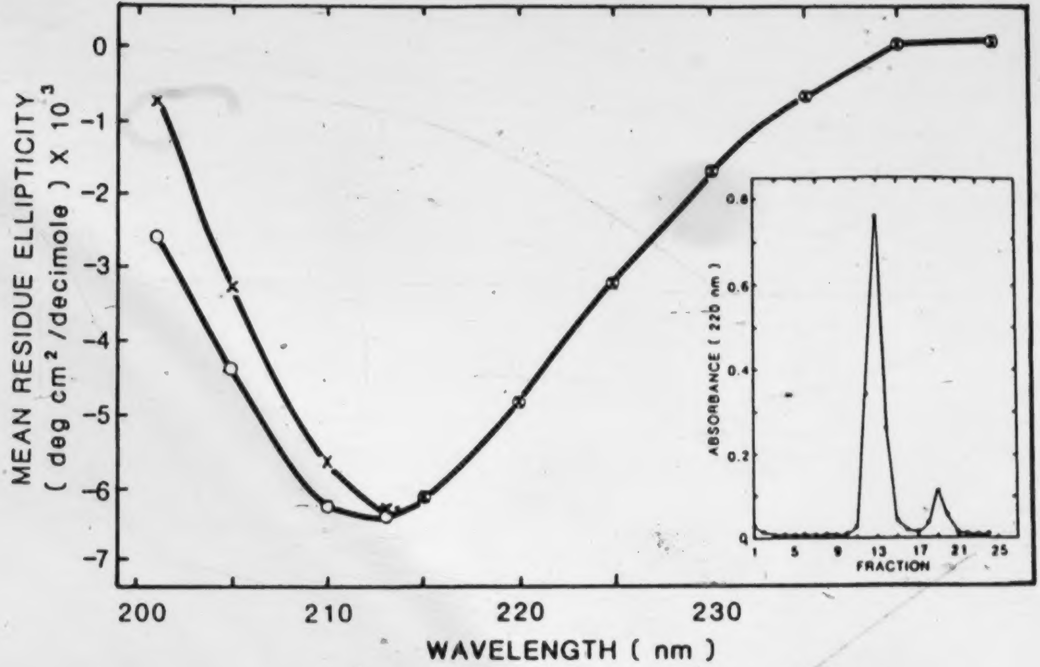

Fig. 4-8. Smoothed far-UV CD spectra of S-protein multimer in distilled water (X) and 3 mM $CaCl_2$ (O). Inset: Analysis by rate zonal centrifugation of the S-protein multimer sample used for circular dichroism measurements.

the *A. vinelandii* S-layer would be classified as an M_4C_4 type.

Distilled water extraction of isolated outer membrane fragments releases primarily a tetrameric form of the 60K protein which constitutes the *A. vinelandii* surface array. The image processing data suggests that the morphological subunit of the array is tetrameric which supports the idea that the multimeric form of S-protein is derived from this structure. Distilled water extraction does not randomly disintegrate the array because only two quaternary forms of S-protein are seen. Thus, tetrameric S-protein must represent either the intact tetrads or intact linkage units. Primarily due to the extreme lability of tetrameric S-protein, the distilled water extracts have not been examined by electron microscopy for evidence of the morphological subunits. However, it is likely that distilled water extraction primarily disrupts the linkage region of the array, producing free tetrads since there appears to be more extensive protein:protein contacts within the tetrad than within the linking structure. In support of this notion, the linking region appears to be the weakest part of the *Synechocystis* S-layer (Karlsson *et al.*, 1983; Vaara, 1982) and the extent of connectivity in the linking region of S-layers is thought to affect their stability (Chalcroft *et al.*, 1986; Rasch *et al.*, 1984). The 'S-layers' of *Pseudomonas acidovorans* (Chalcroft *et al.*, 1986) and *Deinococcus radiodurans* (Baumeister *et al.*, 1982) may be extreme examples of this principle. The former 'S-layer' possesses little or no detectable connectivity between the morphological subunits and not surprisingly this layer cannot be freed intact from the outer membrane. On the other hand, the regions of connectivity in the *D. radiodurans* S-layer appear extremely extensive and correspondingly, this layer is extremely resistant to denaturing agents even when not attached to the underlying cell wall. The array of *A. vinelandii* also appears very labile when detached from the outer membrane requiring the divalent cations in Burk buffer to maintain its integrity during negative staining.

Although image processing methods applied to a number of bacterial S-layers have indicated that morphological subunits of the tetragonal and hexagonal type appear to be composed of four and six smaller subunits respectively (Baumeister *et al.*, 1986; Sleytr and Messner, 1983) in no case has this supposition been confirmed biochemically, although Baumeister *et al.* (1982) did determine the mass of the morphological subunit of the hexagonal S-layer of *D. radiodurans in situ* using scanning transmission electron microscopy and found it to be consistent with a hexamer.

The ability to isolate intact morphological subunits under relatively mild conditions has allowed an evaluation of factors important to the stability of this structure. The morphological subunit is quite unstable once released from the outer membrane, dissociating into monomers when exposed to buffers such as HEPES and potassium phosphate in which it is stable while attached to the cell surface (see Page and von Tigerstrom, 1982). The tetramer could be stabilized with low concentrations of both Mg^{2+} or Ca^{2+} indicating that divalent cations are probably required in the assembly of the morphological subunit. This finding in conjunction with previous results (Bingle *et al.*, 1986; Chapter 3) that divalent cations could mediate *in vitro* reassembly of S-protein onto the surface of cells stripped of their native S-layer shows that divalent cations are also important for attachment of the subunit to the cell surface. The aggregation of the morphological subunit with divalent cations indicates that there must be other cation binding sites in addition to those which are involved with linking the subunit to the outer membrane.

The secondary structure of the morphological subunit is not radically different from that reported for monomeric S-protein (Bingle *et al.*, 1986; Chapter 3). Since the association with the outer membrane clearly stabilizes the array it might be expected that release of the subunits from the outer membrane could cause a perturbation of the protein molecules, leading to the high degree of random coil configuration observed. However, Baumeister *et al.* (1982) reported a similar high degree of random coil structure for intact S-layer fragments from *D. radiodurans,* indicating such aperiodic structure is probably present in the organized S-layer. The term random coil is not meant to imply a true random coil configuration but is used in the context of circular dichroic measurements as that structure not attributable to α-helix or ß-sheet configurations.

Despite the negligible differences in secondary structure between monomeric and tetrameric S-protein the response of the two species to divalent cations is radically different. Monomeric S-protein is not aggregated by Ca^{2+} or Mg^{2+} while tetrameric S-protein is both stabilized and aggregated by these cations. This indicates there may be some conformational differences between the two species which affects the disposition of the cation binding sites although circular dichroism has not revealed it. In support of this idea, while tetrameric S-protein can be stabilized by divalent cations, once dissociated into monomers addition of divalent cations does not induce restoration of the tetramer even if a suitable outer membrane template is present to orient the molecules if necessary.

Assembly of the morphological subunit of the *A. vinelandii* surface array seems to be a one way street, but if preformed morphological subunits are available the array can be induced to recrystallize *in vitro.* Since the quaternary structures of S-proteins extracted from the cell walls of other bacteria is rarely determined, it is not clear in many cases whether *in vitro* reassembly of other S-layers occurs from monomers as the starting material or requires preformed morphological subunits. The former situation is usually considered to be the case due to the concentrations of urea and guanidine hydrochloride used for the extraction of S-layer subunits (Koval and Murray, 1984). In those instances where information is available, there is no universal mechanism. The S-layers of *Aquaspirillum serpens* VHA (Buckmire and Murray, 1973) and *Bacillus stearothermophilus* (Jaenicke *et al.*, 1985) reassemble from monomeric S-protein through to intact S-layer sheets. On the other hand, the reassembly of the S-layer of *Acinetobacter* 199A apparently begins with an aggregated form of this S-layer protein (Thornley *et al.*, 1974).

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5. Role of calcium in the assembly of the *Azotobacter vinelandii* S-layer

5.1 Introduction

Surface layers composed of regularly arranged protein subunits external to the outer membrane, are present on many gram-negative eubacteria. Of the gram-negative genera studied in any detail members of only one, *Aquaspirillum*, have been definitely shown to require divalent cations, specifically Ca^{2+}, for the assembly of their surface layers into regular geometric arrays (Beveridge and Murray, 1976a; Buckmire and Murray, 1976; Kist and Murray, 1984). However, preliminary experiments suggest that the stability of the S-layer of *Caulobacter crescentus* and *Acinetobacter* 199A may be dependent on divalent cations such as calcium and magnesium (Smit *et al.*, 1981; Thorne *et al.*, 1975).

The importance of divalent cations to S-layer assembly may be related to the generally acidic nature of the proteins constituting the arrays (Sleytr and Messner, 1983) and may be an extension of the importance of these species in outer membrane structure. The divalent cation requirements of surface arrays are usually gauged by incubating isolated S-layer protein with cations in the presence of a cell wall template, followed by electron microscopic examination for evidence of the assembly of the protein into a regular array. Through this type of experiment it has been shown that the *Acinetobacter* 199A surface array is relatively non-specific with respect to its divalent cation requirements (Thorne *et al.*, 1975). In contrast, *A. serpens* MW5 (Kist and Murray, 1984) specifically requires Ca^{2+} for S-layer assembly. However, the S-layer of *A. serpens* VHA will self-assemble in the presence of both Mg^{2+} or Ca^{2+} (Buckmire and Murray, 1976). Significantly, it was shown that the Mg^{2+}-reassembled array was susceptible to disruption by distilled water, while the Ca^{2+}-reassembled array was not, a property shared by the native array. Thus, *in vitro* reassembly experiments can give misleading results with respect to *in vivo* assembly requirements, unless the stability of the reassembled array can be compared to the native array.

This chapter further explores the importance of divalent cations, specifically Ca^{2+} and Mg^{2+}, in the assembly of the *A. vinelandii* surface layer. This study was prompted by the

A version of this chapter has been published. Doran, J.L., W.H. Bingle and W.J. Page. 1987. Role of calcium in the assembly of the *Azotobacter vinelandii* surface array. J. Gen. Microbiol. 133:399-413.

observations that *A. vinelandii* grown in Ca^{2+}-limited medium did not possess an S-layer visible by freeze-etch electron microscopy (W.H.Bingle, J.L. Doran, and W.J. Page, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, K155, p. 202) although adequate Mg^{2+} was present in the medium for assembly of the protein into a regular array (Bingle *et al.*, 1986; Chapter 3).

5.2 Materials and Methods

Bacterial strains and growth conditions

A. vinelandii UW1 was grown in a potassium phosphate based minimal medium (Burk medium) containing 0.6 mM $CaCl_2$ and 0.8 mM $MgSO_4$ (Bingle *et al.*, 1984; Chapter 2). Ca^{2+}-limited (0Ca) medium contained all components of Ca^{2+}-sufficient (+Ca) medium except $CaCl_2$. Water was either glass double distilled or treated with the Milli-Q water purification system (Millipore Corporation, El Paso TX). For inoculation of Ca^{2+}-limited liquid medium, 0Ca cells were pregrown for 24 h on 0Ca medium solidified with 1.8% agar. Liquid cultures were inoculated to an initial optical density of 0.05 and were grown at 30°C in a gyratory water bath shaker operating at 175 rpm. The cultures were harvested when the culture optical density (620 nm) reached between 1.2 and 1.5 (a Bausch and Lomb Spectronic 20 was used for optical density measurements). Analysis of 0Ca medium by atomic absorption spectroscopy (Greenburg *et al.*, 1985) indicated no detectable Ca^{2+} in excess of levels present in double distilled or Milli-Q treated distilled water. 0FeBB and $BBPO_4$ have been defined previously (Bingle *et al.*, 1986; Chapter 2).

Freeze-etch electron microscopy

In order to expose regular arrays on the cell surface assembled *in vivo*, it was necessary to wash the cells at an elevated temperature prior to freeze-etching. Cells were resuspended at an OD_{620} of 1 in $BBPO_4$ at 42°C and incubated at 42°C for 5 min. The cells were collected by centrifugation and the wash treatment was repeated an additional four times. When appropriate, chloride salts of the divalent cations-Mg^{2+}, Ca^{2+}, Be^{2+} and Sr^{2+} were included in $BBPO_4$ at a concentration of 0.5 mM. After washing, the cells were resuspended in residual buffer following decantation and processed for freeze-etch electron microscopy as previously described (Bingle *et al.*, 1984; Chapter 2). The above washing protocol was not used when visualizing arrays reassembled *in vitro* from isolated S-protein.

Recovery of protein and lipopolysaccharide from distilled water wash fluids, culture supernatant and EDTA or EGTA extracts

Cells were washed with distilled water to remove the surface array by a modification of the filtration method of Schenk and Earhart (1981) as outlined by Bingle *et al.* (1984). Distilled water wash fluids and culture supernatants were initially filtered through a 0.45 µm pore size Millipore filter. Protein released into these fluids was recovered by ultrafiltration using an Amicon PM-10 membrane (molecular weight cut-off, 10,000). Lipopolysaccharide (LPS) was recovered from wash fluids and culture supernatant by lyophilization followed by dialysis against distilled water (molecular weight cut-off, 3500). Before lyophilization ethylenediamine tetraacetic acid (EDTA) was added to a concentration of 2 mM to culture supernatants. Protein:LPS complexes were released from whole cells resuspended in 10 mM Na^+-HEPES with 2 mM EDTA or ethyleneglycol-bis-(ß-aminoethylether) N,N'-tetraacetic acid (EGTA).. The cells were washed in one volume of 10 mM K^+-HEPES, pH 7.5 and resuspended in one volume of Na^+-HEPES/EDTA or EGTA, pH 8, followed by incubation for 1 h at 30°C with slow shaking. The cells were removed by centrifugation (15,000 g, 10 min, 4°C) and the supernatant was filtered through a 0.8 µm pore size Millipore filter prior to concentration by lyophilization. The concentrated extracts were dialyzed (molecular weight cut-off, 3500) against distilled water at 4°C.

Protein was estimated either by the method of Bradford (1976) using bovine gamma globulin as a standard or by a modification of the Lowry method (Markwell *et al.*, 1978) using bovine serum albumin (BSA) as a standard. The concentration of 2-keto-3-deoxyoctanoate (KDO) was determined by the method of Keleti and Lederer (1974). When determining KDO in whole cell extracts, the cells were resuspended in distilled water and broken in the French pressure cell. Granules of poly-ß-hydroxybutyrate (PHB) were removed by low speed centrifugation (2000 g, 20 min) and the extract was dialyzed (molecular weight cut-off, 3500) overnight against 1000 volumes of distilled water at 4°C.

Isolation of outer membranes and extraction of S-protein

Cells were disrupted using the French pressure cell and outer membrane fragments were recovered on sucrose gradients essentially according to the method of Page and von Tigerstom (1982) with one modification: Tris buffer was replaced with 10 mM K^+-HEPES, pH 7.5. The refractive index of the sucrose gradient fractions was determined with an Erma refractometer (Erma Optical Works Ltd., Tokyo, Japan). S-protein was extracted from outer membrane fragments with distilled water as described by Bingle *et al.* (1986; Chapter 3).

The proportion of S-protein in tetrameric and monomeric form was determined by rate zonal centrifugation in linear glycerol gradients (6-30% (v/v) glycerol in 10 mM K^+-HEPES, pH 7.5) as outlined by Bingle *et al.* (1986; Chapter 3).

Cross-linking of S-protein

Cell walls were purified on sucrose gradients after disruption of the cells using the French pressure cell by the method of Page and von Tigerstrom (1982). Two modifications were made to the protocol: 0FeBB buffer was substituted for Tris/HCl buffer and the lysozyme treatment was omitted. Purified outer membranes were recovered on a cushion of 72% sucrose by centrifugation at 200,000 g for 1 h and used directly for cross-linking after adjustment of the protein concentration of the suspension to 5 mg/ml (Markwell *et al.*, 1978). Free S-protein multimer (1 mg/ml) was isolated by extracting outer membrane pellets (3 mg outer membrane protein each) with 1 ml of distilled water (Bingle *et al.*, 1986; Chapter 3). Outer membranes were cross-linked at a concentration of 500 μg/ml protein while free S-protein multimer was treated at a concentration of 100 μg/ml.

Dimethyl suberimate or DMS (Davies and Stark, 1970) and 3,3'-dithiobispropionimidate or DTP (Wang and Richards, 1975) were freshly prepared in 50 mM Na_2HPO_4, pH 10 followed by quick readjustment of the pH to 8.8. Cross-linking of cell wall proteins was done in at least 50 mM Na_2HPO_4 while at least 10 mM Na_2HPO_4 was used for S-protein samples. The initial pH of the reaction mixtures was 8.8 and cross-linking proceeded for 15 min at 33°C at which time the reactions were stopped by adding a two-fold molar excess of Tris/HCl, pH 8.8. Cross-linked proteins were recovered on ultrafiltration membranes using the Amicon micropartition-ultrafiltration system (Amicon Corp., Oakville, Ontario) and resuspended in 62.5 mM Tris/HCl, pH 6.8. Sodium dodecyl sulfate (SDS) was added to 2% (w/v) and the cross-linked products were analyzed by SDS-PAGE.

Probes of S-protein conformation

The conformation of S-protein on the surface of cells grown in the presence and absence of Ca^{2+} was investigated by determining its susceptibility to radioiodination and to proteases of differing specificities. The accessibility of tyrosine residues of surface localized S-protein to radioiodination (50 µg Iodo-Gen and 0.5 µCi/µl ^{125}I) was examined as described by Bingle *et al.* (1984) with one modification: after radioiodination, the cells were washed with buffer containing 1 mM NaI not 25 mM NaI.

The sensitivity of surface localized S-protein of +Ca and OCa cells to the proteases (Sigma Chemical Co., St. Louis MO) trypsin, *Staphylococcus aureus* V8 protease, and thermolysin was examined. EGTA was added in equimolar amounts to the Ca^{2+} present in the thermolysin preparation prior to use. A 1.5 ml aliquot of cells was collected by centrifugation using a Fisher model 235 microfuge and the pellet was resuspended in 1 ml of OCa buffer and freshly prepared protease was added. The suspension was incubated for 1 h at 30°C at which time the cells were pelleted again and resuspended in the appropriate buffer containing the following protease inhibitors: trypsin inhibitor from soybean (trypsin), 0.1 mM diisopropylflurophosphate (*S. aureus* V8 protease) and 0.1 mM 1, 10 phenanthroline (thermolysin) and incubated for 1 h. The cells were pelleted again, resuspended in 1 ml of 0.1% sodium lauroyl sarcosine (Sarkosyl) and vortexed vigorously for 1 min at room temperature to extract S-protein from the cell surface. The detergent extract was immediately frozen in a 1.5 ml Eppendorf tube, lyophilized and the residue was resuspended in 100 µl distilled water for analysis by SDS-PAGE.

Analysis of cell wall fractions for Ca^{2+} and Mg^{2+}

Cells were cultivated in OCa or +Ca medium as described above except the aeration of the culture was enhanced by increasing the shake speed to 300 rpm. S-protein extracted with distilled water from purified cell walls was concentrated by ultrafiltration and then collected by centrifugation on a YMT ultrafiltration membrane (molecular weight cut-off, 10,000). The protein was resuspended from the membrane in double distilled deionized water. Samples (10-12 mg dry weight cell walls and 5-8 mg dry weight S-protein) were first digested with 2 ml concentrated HNO_3 and heated to dryness at 110°C. The residue was redissolved in 0.5 ml concentrated HCl and diluted to 10 ml with distilled water. The residual cell wall material lacking S-protein was lyophilized and treated in a similar manner. Lanthium was added to a final concentration of 2 g/l prior to analysis. The Ca^{2+} and Mg^{2+} content of the samples was determined by atomic absorption spectroscopy using an Instrument Laboratory aa/ae spectrophotometer 751 according to Greenburg *et al.* (1985). A control sample containing all reagents was assayed for background Ca^{2+} and Mg^{2+} levels.

Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted according to Laemmli (1970) in 10% polyacrylamide gels using apparatus and conditions outlined by Bingle *et al.* (1984; Chapter 2). The molecular weight of cross-linked products in excess of 100K were determined using 7% polyacrylamide gels and the following molecular weight standards (Sigma): myosin (205K), ß-galactosidase (116K), phosphorylase B (97.4K), bovine serum albumin (66K). Gels were stained for protein and carbohydrate by the method of Fairbanks *et al.* (1971). For densitometry, the quantitative staining method of Blakesley and Boezi (1977) with Coomassie brilliant blue G-250 was used in conjunction with a A Hoeffer GS-300 scanning densitometer (Hoefer Scientific Instruments, San Francisco, CA). The peak area of the S-protein band relative to the amount of protein loaded per SDS-PAGE sample well was linear between 1 and 8 µg of protein.

5.3 Results

Freeze-etch electron microscopy of cells grown in 0Ca medium

When *A. vinelandii* grown in Ca^{2+}-limited medium was examined for the presence of the regular S-layer by freeze-etch electron microscopy, it was not evident (Fig. 5-1); cells grown in the presence of Ca^{2+} exhibited the typical tetragonal array. The outer surface of 0Ca cells exhibited a stippled appearance (not reported by Doran, 1983) which was limited to the external surface and was not evident on the outer membrane fracture face or the inner

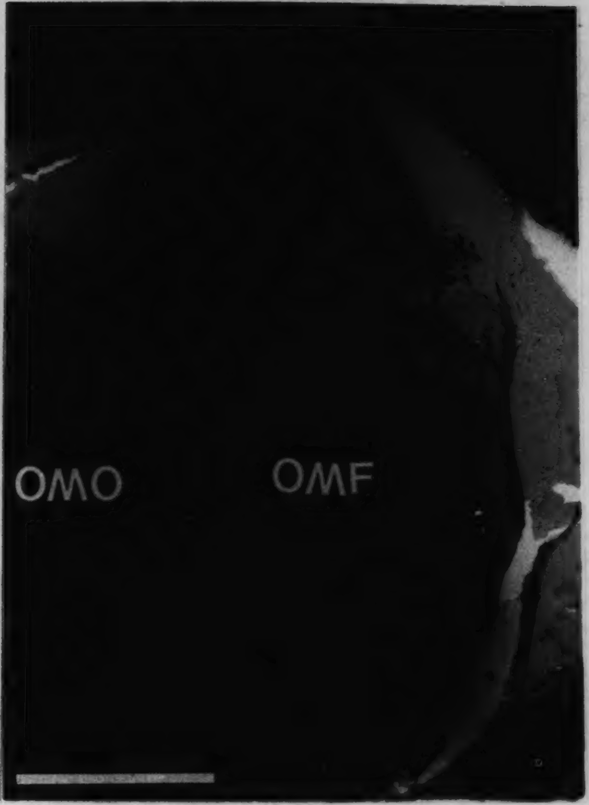

Fig. 5-1. Freeze-etch replica of the convex surface of *A. vinelandii* UW1 grown in Ca^{2+}-limited medium. Abbreviations: S, S-layer; OMF, outer membrane fracture face; OMO, outer surface of the outer membrane. Bar, 0.5 µm.

membrane fracture face. When an aliquot of culture was made 0.5 mM with $CaCl_2$ and incubated at 30°C for 1-2 h the regular array could be found covering the entire cell surface (Fig. 5-2) . Sr^{2+} could substitute for Ca^{2+} however when additional Mg^{2+} was added to the culture, the regular array did not reappear. This indicated that the lack of the array was not due to a general deficiency of divalent cations but was specifically due to a lack of Ca^{2+}. Similarly, addition of Be^{2+} did not mediate the reappearance of the S-layer. In each case described above, after incubation with the divalent cation the cells were washed with $BBP0_4$ containing the appropriate cation as described in Materials and Methods. To preclude unforeseen artifacts from the washing procedure, the order of the cation addition and washing steps was reversed; identical results were obtained. In this case, the cells were washed with OCa buffer and then exposed to each divalent cation.

Outer membrane protein profiles of 0Ca cells

The effect of Ca^{2+} on S-layer assembly, when added to cells grown in OCa medium, could have involved a requirement for *de novo* synthesis and/or translocation of the nascent or completed polypeptide to the cell surface. These possibilities were eliminated by examining the polypeptide composition of outer membranes isolated from 0Ca cells (Fig. 5-3; lane 1). Such cells possessed S-protein which copurified with the outer membrane fraction. The amount of S-protein did not appear reduced from that normally seen for Ca^{2+}-sufficient cells (Bingle *et al.*, 1984; Chapter 2). Secondly, S-protein was extractable from the outer membrane of intact cells by the relatively mild filtration washing method of Schenk and Earhart (1981) indicating it was surface localized (Fig. 5-3; lane 2 and 3). Examination of distilled water washed cells by freeze-etch electron microscopy showed that the stippled appearance of the outer surface had been eliminated producing a relatively smooth surface (Fig. 5-4).

Although S-protein could be removed from both Ca^{2+}-sufficient and Ca^{2+}-limited cells by distilled water washing, the level of Ca^{2+} in the growth medium determined the effectiveness of the distilled water washing protocol with respect to S-protein release. Below 0.5 mM almost complete removal of S-protein could be reproducibly achieved (Fig. 5-5a; lanes 1-4) but above 0.5 mM only approximately 50% of S-protein was released from the outer membrane (Fig. 5-5a; lane 6). A concentration of 0.5 mM Ca^{2+} in the growth medium was a critical level and the amount of S-protein released varied substantially between experiments (Fig 5-5a; lane 5). This dependence of growth medium

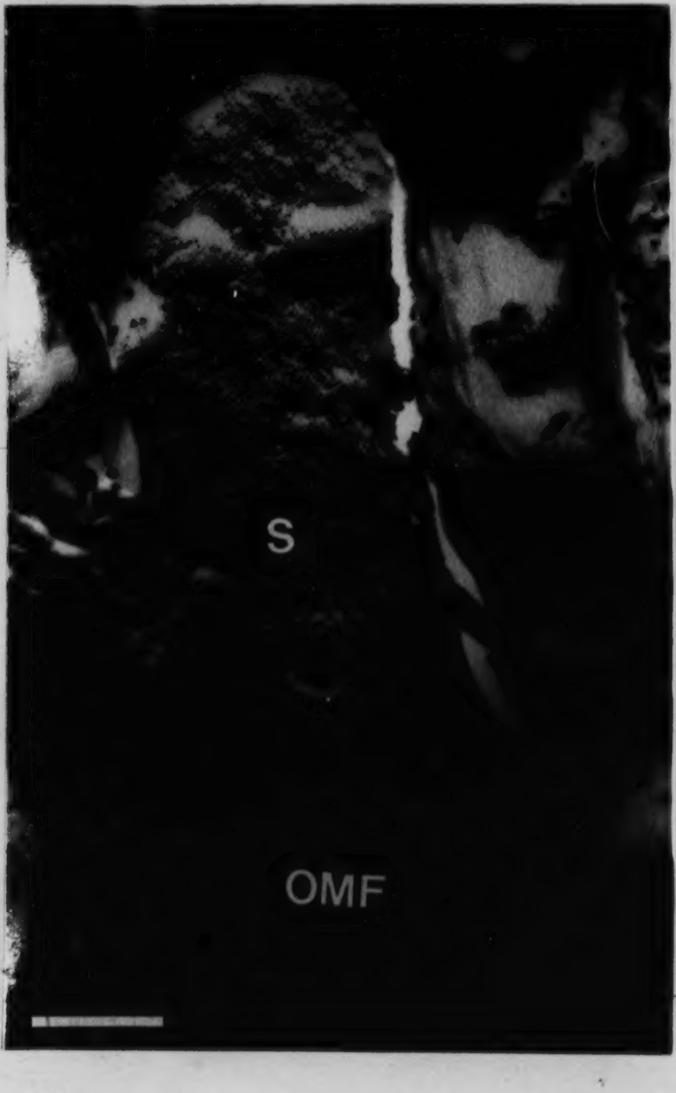

Fig. 5-2. Alteration in the surface features of Ca^{+2}-limited *A. vinelandii* UW1 resulting from incubation with 0.5 mM Ca^{2+} for 1 h at 30°C. Freeze-etch replica; Bar, 0.5 μm. Abbreviations same as Fig. 5-1.

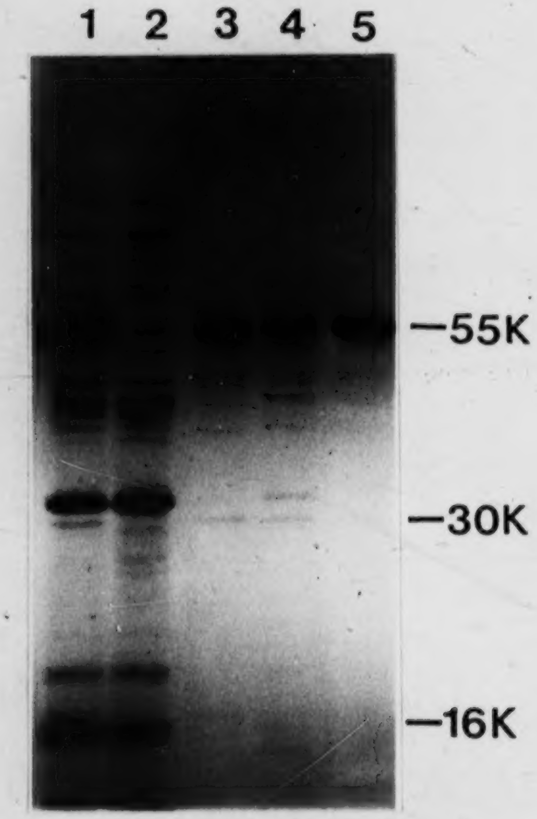

Fig. 5-3. Release of S-protein from intact Ca^{2+}-limited *A. vinelandii* UW1 cells by distilled water extraction. Lanes: 1, outer membrane from unwashed cells; 2, outer membrane from distilled water washed cells; 3, proteins released into distilled water wash fluids; 4, concentrated culture supernatant protein from Ca^{2+}-limited cells; 5, concentrated culture supernatant protein from Ca^{2+}-sufficient cells.

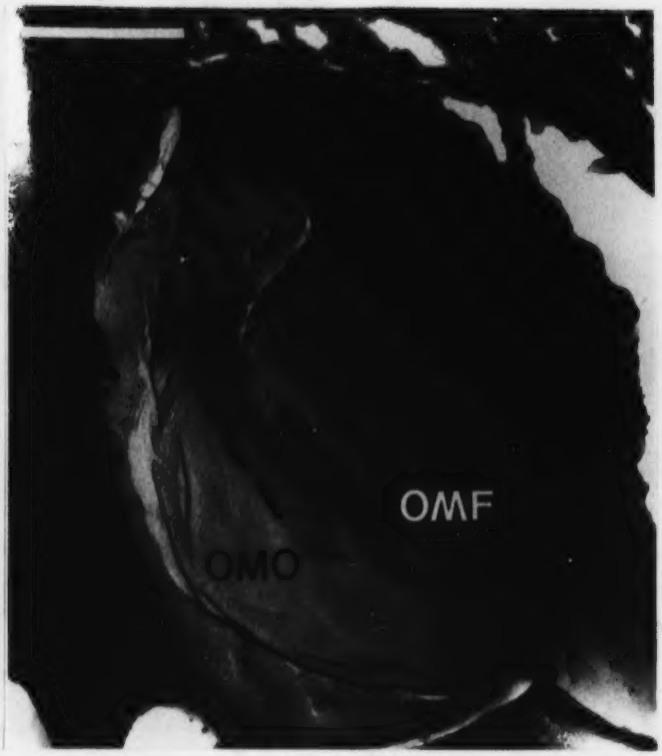

Fig. 5-4. Replica of the convex surface of Ca^{2+}-limited *A. vinelandii* UW1 previously washed with distilled water. Abbreviations same as Fig. 5-1. Bar, 0.5 µm.

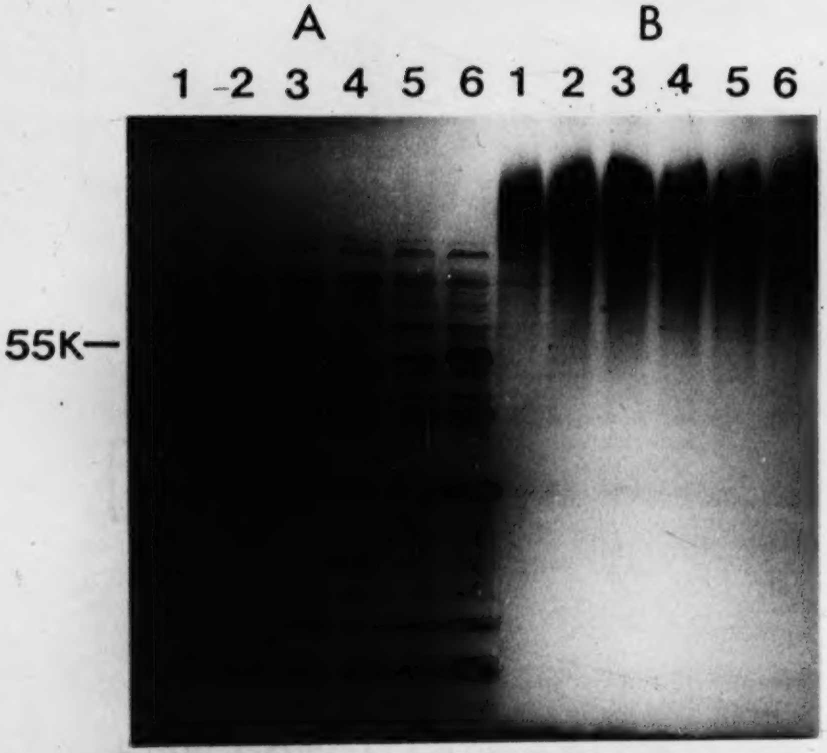

Fig. 5-5. Effect of growth medium Ca^{2+} concentration on the protein and LPS composition of outer membranes isolated from distilled water washed cells. (A) Coomassie blue stained outer membrane proteins. (B) Periodic acid-Schiff stain for LPS. Cells were grown in Ca^{2+}-limited medium (lane 1) or in medium containing 0.1 mM Ca^{2+} (lane 2), 0.2 mM Ca^{2+} (lane 3), 0.3 mM Ca^{2+} (lane 4), 0.5 mM Ca^{2+} (lane 5), or 0.7 mM Ca^{2+} (lane 6).

Ca^{2+} concentration on S-protein release was noted when a new lot of $CaCl_2$ was used for the experiments described in this chapter. Previously it was reported that S-protein could be extracted by distilled water washing of whole cells grown in Burk medium containing 0.6 mM $CaCl_2$ (Bingle *et al.*, 1984; Chapter 2). However, for reproducible results, a concentration of $CaCl_2$ of slightly less than 0.5 mM should be used in the growth medium. SDS-PAGE analysis of the distilled water wash fluids from OCa cells revealed that in contrast to Ca^{2+}-sufficient cells (Bingle *et al.*, 1984; Chapter 2), trace amounts of several additional polypeptides were released. (Fig. 5-3; lane 3). To evaluate whether serious disruption of the outer membrane of OCa cells had occurred during distilled water washing the level of KDO, an LPS marker, was assayed in the concentrated distilled water wash fluids. OCa cells released ten times more KDO/mg protein than +Ca cells, and the amount of KDO released declined with Ca^{2+} content of the medium (Fig. 5-6). Quantitatively, this level amounted to 10% of the total cellular LPS.

Origin of reassembled S-protein

The above results suggested that the stippled appearance of the outer membrane was due to S-protein in an alternate organization and that Ca^{2+} specifically caused its reorganization into a regular tetragonal array. In order to confirm this hypothesis, alternate origins for the newly appearing S-protein were sought. The absence of Ca^{2+} did not cause the S-protein to be more susceptible to loss into the culture supernatant which subsequently reassembled onto the surface of the cells during the incubation with Ca^{2+}. Culture supernatant (1140 ml) from +Ca and 0Ca cells was concentrated by ultrafiltration, analyzed by SDS-PAGE and the amount of protein in the S-protein band was quantified using densitometry. This analysis revealed that identical levels of S-protein were released into the culture fluids regardless of Ca^{2+}-limitation. These data indicated that the appearance of the S-layer upon incubation with Ca^{2+} in culture supernatant was not coming from S-protein released into the culture fluids. This was confirmed by performing the incubations with Ca^{2+}, in fresh buffer, where no supernatant components were present. Cells so incubated also recovered their S-layer.

A final possibility (and an improbable one) was that the pool of S-protein in the outer membrane was purged and the S-layer was synthesized from new protein. This was unlikely because the formation of the S-layer in this non-nitrogen fixing strain of *A. vinelandii* occurred in non-nutrient buffer in the absence of a nitrogen and an external

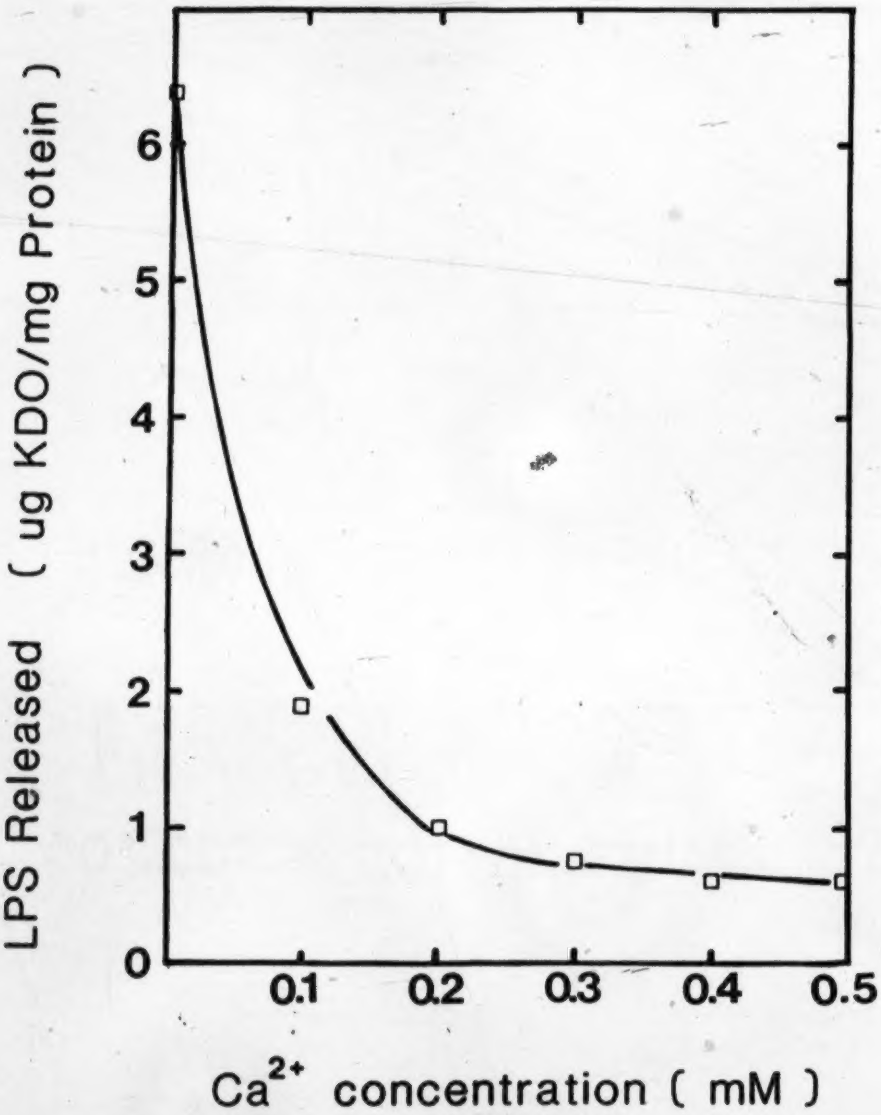

Fig. 5-6. Effect of growth medium Ca^{2+} concentration on the release of LPS (as KDO) by distilled water washing of intact *A. vinelandii* UW1 whole cells. Distilled water wash fluids were concentrated by lyophilization and assayed for protein and KDO by the methods of Markwell *et al.* (1978) and Keleti and Lederer (1974) respectively.

carbon source although the cell probably possessed reserves of carbon as PHB. This was investigated by incubating cells in buffer with $^{35}SO_4^{2-}$ (1 mCi/100 ml culture) during Ca^{2+}-mediated recovery of the S-layer. Subsequently, surface localized S-protein was extracted from the cells with distilled water and the incorporation of ^{35}S into the protein was determined. Using the total protein recovered and the known quantities of sulfur containing amino acids, the proportion of newly synthesized S-protein was estimated. It was found that after the incubation with Ca^{2+}, 10% of the distilled water extractable S-protein could be accounted for as new synthesis. In an attempt to complement this biochemical evidence, OCa cells were incubated in buffer with added Ca^{2+} for 2 h in the presence of 100 µg/ml chloramphenicol. However, when cells so treated were examined by freeze-etch electron microscopy, anomalous fracturing of the cells was observed leaving no visible areas of cell surface. Sleytr and Thornley (1973) noted, using thin-sectioned material, that incubation of *Acinetobacter* 199A with chloramphenicol produced alterations in the cell wall structure and accumulation of extracellular material, which obscured the tetragonal surface layer of this organism. Whether similar alterations in the cell wall of *A. vinelandii* could affect the fracture plane is unknown. Despite this lack of electron microscopic support, the ^{35}S-labelling experiment confirmed that the vast majority if not all of the S-protein assembling into the regular array upon incubation with Ca^{2+} was already synthesized and surface localized.

Involvement of Ca^{2+} in outer membrane structure

Since S-protein was already surface localized, there were two obvious locations in the cell wall which could have been affected by Ca^{2+} addition. Ca^{2+} could have been acting directly on the intermolecular interactions of the S-protein, or indirectly, through an effect on the underlying template layer, the outer membrane. The involvement of Ca^{2+} in outer membrane stability was assessed by incubating cells with EGTA and EDTA. The amount of protein and KDO released from cells treated with these chelating agents was determined (Table 5-1). Cells grown in the presence of 0.6 mM Ca^{2+} were nearly as sensitive to EGTA as EDTA releasing about 90% as much LPS with EGTA as that released by EDTA. Similarly, the amount of protein released by EGTA amounted to 75% of that released by EDTA. SDS-PAGE of EGTA and EDTA extracts showed numerous protein species were released (Fig. 5-7A; lanes 1 and 2). The presence of LPS and polypeptides of higher molecular weight than S-protein indicated that the protein bands of molecular weight lower than S-protein were unlikely to be only degradation products of S-prottein

Table 5-1. EDTA and EGTA mediated release of protein and LPS from Ca^{2+}-sufficient and Ca^{2+}-limited *A. vinelandii* UW1.

	Protein released (% total cell protein)		LPS released (% total cell KDO)	
Treatment	+Ca	OCa	+Ca	OCa
2 mM EDTA	4.3 (0.7%)	9.8 (0.3%)	60 (17%)	53 (4.3%)
2 mM EGTA	3.3 (0.7%)	1.8 (0.1%)	54 (4.7%)	23 (1.5%)

Numbers in brackets indicate standard deviation of two replicate determinations.

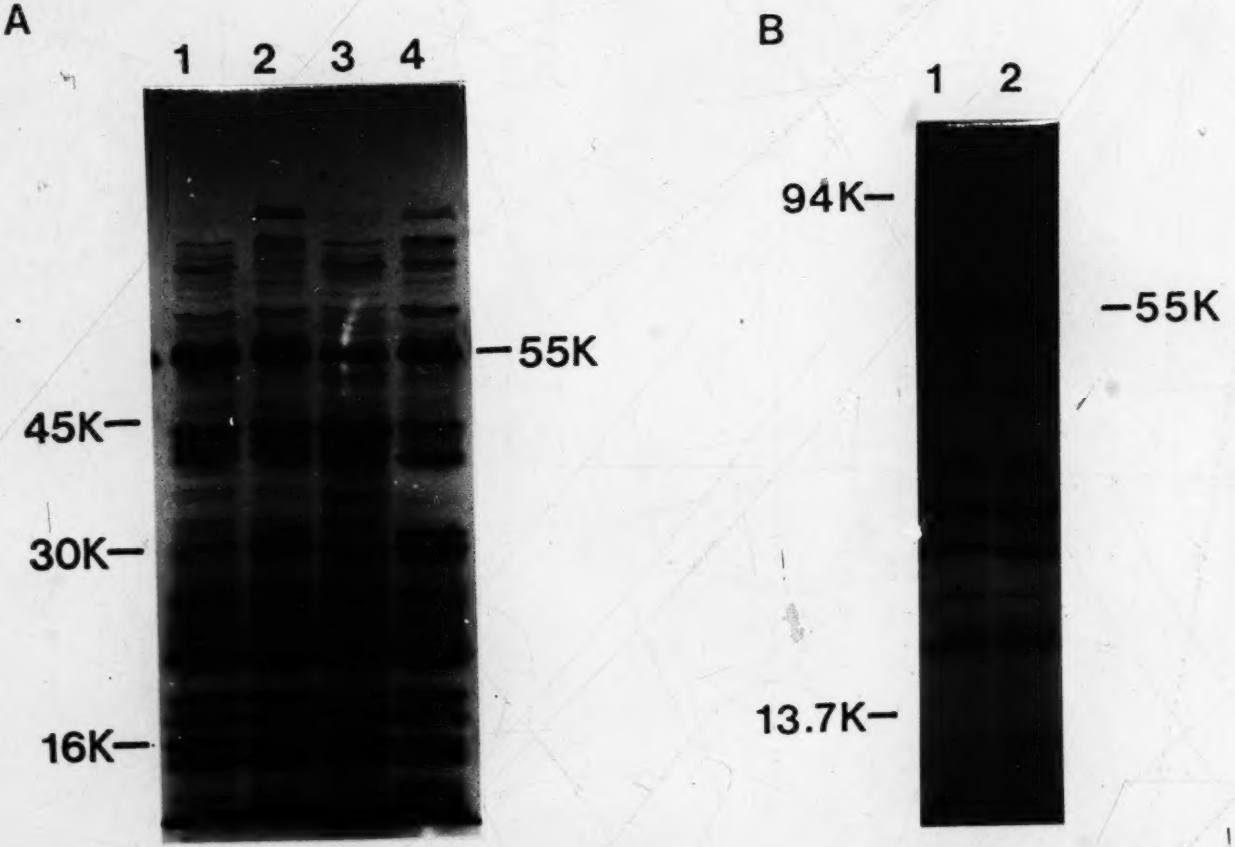
A
1
2
3
4
—55K
45K—
30K—
16K—
B
1
2
94K—
—55K
13.7K—

Fig. 5-7. (A) SDS-PAGE of proteins released from Ca^{2+}-sufficient (lanes 1 and 2) and Ca^{2+}-limited (lanes 3 and 4) cells of *A. vinelandii* UW1 with 2 mM EDTA (lanes 1 and 3) or 2 mM EGTA (lanes 2 and 4). (B) Effect of 2 mM EDTA (lane 1) or EGTA (lane 2) on the release of proteins from osmotically stabilized *A. vinelandii* UW1 Ca^{2+}-sufficient whole cells.

(see Koval and Murray, 1984) but were due to a general disruption of the cell wall by EGTA and EDTA. EDTA and EGTA treatment of osmotically stabilized cells (15% w/v sucrose) showed that an identical subset of proteins was released by the two chelating agents (Fig. 5-7B).

The sensitivity of the cell wall to EGTA suggested a role for Ca^{2+} in outer membrane structure. If so, the sensitivity of 0Ca cells to EGTA should be lower than +Ca cells due an absence of Ca^{2+}, or a substitution of Mg^{2+}, in some sites of the outer membrane (Boggis *et al.*, 1979). This was found to be the case (Table 5-1) as indicated by a 50% reduction in LPS and protein released from Ca^{2+}-limited cells with EGTA as compared to Ca^{2+}-sufficient cells. The effect of EDTA on Ca^{2+}-limited cells as compared to Ca^{2+}-sufficient cells was difficult to gauge. Although both types of cells released the nearly the same amount of LPS, Ca^{2+}-limited cells released two-times as much protein (Table 5-1). If one postulates that the site of attack of EDTA and EGTA is primarily LPS (Hancock, 1984) then extraction of this species from Ca^{2+}-limited cells apparently caused more destabilization of the cell envelope and consequently a larger release of protein as compared to Ca^{2+}-sufficient cells. The proteins released from these cells also appeared more diverse (Fig. 5-7A; lanes 3 and 4).

These experiments pointed to a role for Ca^{2+} in the structure of the outer membrane. If Ca^{2+} was important for envelope integrity increased levels of both protein and LPS should be evident in culture supernatant from OCa cells. Ca^{2+}-sufficient cells released 0.8 μg/ml protein into the culture fluids whereas Ca^{2+}-limited cells released 1.5 μg/ml. Analysis by SDS-PAGE revealed that S-protein was the only protein present in Ca^{2+}-sufficient culture fluids while several additional minor proteins were found in Ca^{2+}-limited culture fluids (Fig. 5-3; lanes 3 and 4). Although two times as much protein was released into Ca^{2+}-limited culture fluids, it was clear from SDS-PAGE and densitometry this increase was not due to increased release of S-protein but to the accumulated effect of many minor species. No thiobarbituric acid positive material, ie. KDO, could be detected in Ca^{2+}-sufficient culture supernatants while a level of 0.25 μg/ml was found in Ca^{2+}-limited culture supernatants. This amounted to 13% of that associated with the cells. The absorption spectrum of the chromogen indicated it was derived from KDO and analysis by SDS-PAGE, followed by periodic acid-Schiff (PAS) staining showed PAS positive bands exhibiting size heterogeneity in the same region of the gel as LPS from SDS solubilized outer membrane samples.

Despite the increased release of LPS due to Ca^{2+} limitation, PAS staining revealed little alteration in the LPS profile of the outer membrane (Fig. 5-5b). The LPS of *A. vinelandii* appeared to be composed of a major high molecular weight fraction of limited size heterogeneity similar to that recently reported for *Aeromonas salmonicida* (Evenberg *et al.*, 1985). Secondly, the yield of outer membrane (expressed as protein recovered) from equivalent volume of culture was unaffected by the presence or absence of Ca^{2+} in the growth medium and outer membranes isolated from both +Ca and 0Ca cells banded at the same position on sucrose gradients indicating both had a density of 1.27 g/cm^3 and that the protein:LPS ratio of the two types of outer membranes were not significantly different.Finally, the omission of Ca^{2+} from the culture medium did not appear to have any gross effect on the growth of *A. vinelandii* UW1; both cultures synthesized the same level of total protein (~200 µg/ml) over the 18-20 h growth period. Cultures grown in the presence of Ca^{2+} became slightly iron-limited as evidenced by low production of the siderophore azotobactin (Page and Huyer, 1984) whereas Ca^{2+}-limited cells did not. This was probably due to the formation of Ca/Fe phosphate co-precipitates which rendered iron more insoluble in culture media containing Ca^{2+}. Although the experiments described above showed that Ca^{2+}-limitation produced somewhat 'leaky' cells, the gross behaviour of calcium-limited cultures suggested that growth and outer membrane integrity were not seriously compromised.

In order to confirm that Ca^{2+}-limitation had not produced an outer membrane incompatible with organization of the S-layer subunits into a tetragonal array, S-protein subunits isolated from outer membranes of +Ca cells were incubated in the presence of Mg^{2+} alone, using distilled water washed OCa cells as a template for reassembly. Subsequent examination by freeze-etch electron microscopy showed that S-protein subunits could organize into a tetragonal array on this surface without the requirement for additional Ca^{2+}. This confirmed that the surface was competent to support organization of the S-layer and that the inhibitory effect of Ca^{2+}-limitation on S-layer assembly was unlikely to reside in the outer membrane.

S-layers reassembled *in vitro* and their stability

The ability of Ca^{2+} (or Sr^{2+}), and not Mg^{2+}, to support reassembly of the S-layer when S-protein was localized on the cell surface *in vivo,* seemed to conflict with previous results which showed that Mg^{2+} could promote reassembly of externally added S-protein onto the surface of distilled water washed cells. In order to determine whether Mg^{2+}-mediated *in vitro* reassembly of the S-layer had returned it to its native state, the stability of S-layers reassembled with Mg^{2+} was tested by incubating whole cells at 42°C in 5 mM potassium phosphate buffer, pH 7.2 for 1 h. Previous experiments (Bingle *et al.*, 1986; Chapter 3) showed that monovalent cations did not support S-layer reassembly at concentrations near 5 mM while the native array assembled *in vivo* was stable to incubation in $BBPO_4$ at 42°C. (Bingle *et al.*, 1984; Chapter 2). When cells carrying Mg^{2+}-reassembled S-layers were treated as described above, and examined by freeze-etch electron microscopy for evidence of the tetragonal array, it was absent. Concentration of supernatant fluids from the heat treated cells and analysis by SDS-PAGE indicated that this treatment caused release of S-protein from the cell surface. Protein assay confirmed that after heat treatment in potassium phosphate buffer, all S-protein that had originally reassembled onto the surface of the distilled water washed cells could be recovered in the supernatant fluids. Thus, Mg^{2+}-mediated reassembly had not returned the S-layer to its native state. Surprisingly, when parallel experiments were carried out with Ca^{2+}-reassembled S-layers, the same result was observed. Regardless of the cation species used, the array was not returned to its native state when S-protein was reassembled onto the surface of distilled water washed cells. In contrast to the results with reassembled S-layers, control experiments confirmed that the native array assembled *in vivo* was stable to this treatment. Two possibilities were suggested by these experiments: (1) divalent cation mediated *in vitro* reassembly of the *A. vinelandii* S-layer was a completely artifactual process or (2) some other stabilizing interactions, in addition to salt bridging, were present in the native array assembled *in vivo* which could not be duplicated when the array was assembled in a vectorially opposite manner to that naturally employed by the cell. The first possibility was unlikely because the omission of Ca^{2+} from the medium clearly caused disorganization of the S-layer. Thus, it appeared that unlike S-protein added externally to naked cells, S-protein exported to the cell surface *in vivo* became associated with the outer membrane in a manner such that only Ca^{2+} could promote organization of the protein into a tetragonal array.

Cross-linking of S-protein

In order to determine if a close association existed between S-protein and an outer membrane protein, bifunctional cross-linking reagents were used. All the expected cross-linked products could be examined on 7-10% polyacrylamide gels. Cross-linking of cell wall proteins was first attempted with dithiobis(succinimidyl) propionate (DSP) by the method of Reithmeier and Bragg (1977) with cell walls suspended in OFeBB. However, only a minimal reduction (if any) in the S-protein band running in the 55-60K region of

SDS-gels was noted, indicating minimal cross-linking of S-protein. In contrast, integral outer membrane proteins were cross-linked into high molecular weight complexes which did not even enter the stacking gel; this agreed with the literature (Angus *et al.*, 1983; Reithmeier and Bragg, 1977). The use of extended (and unrealistic) reaction times (8 h) and 1 mg/ml DSP incubated with 500 μg of cell wall protein did not improve the reactivity of S-protein.

In order to maintain the solubility of DSP in excess of 1 mg/ml in aqueous buffers, increasing amounts of polar organic solvents are required (Lotmant and Fairbanks, 1976) which may perturb the membrane, thus the useful concentration range of the reagent is limited. Although imidoesters are inferior cross-linking reagents because of their notoriously short half-lives and side reactions they are readily soluble (Browne and Kent, 1975). Experiments with DSP seemed to indicate that reagent concentration was limiting, not reaction time. Treatment of isolated cell walls (outer membrane and associated peptidoglycan) with increasing concentrations of DMS produced an obvious modification of S-protein (Fig. 5-8). Even at the lowest concentrations of DMS used, a smearing of the S-protein band in the 55-60K region of the gel was produced. It was clear that although DMS concentrations of 1-2 mg/ml produced little effect on S-protein beyond an increase in the apparent molecular weight, most integral outer membrane proteins were cross-linked into high molecular weight complexes which did not enter the stacking gel. The major 35K and 44-45K outer membrane proteins of *A. vinelandii* (Page and von Tigerstrom, 1982) were almost eliminated from the gels at these DMS concentrations.

These data indicated that integral outer membrane proteins were cross-linked out from under the S-layer. At concentrations of cross-linker in excess of 5 mg/ml a major cross-linked species was produced with an apparent molecular weight of 160K concomitant with a loss of S-protein from the 55-60K region of the gel. In an effort to confirm that this cross-linked species was formed from S-protein only, a cleavable analogue of DMS, DTP, was substituted in the reaction mixtures. However, the high molecular weight cross-linked species could not be generated using this reagent. Evidence of extensive side-reactions with this reagent, including the formation of polymeric sulfur compounds was noted, preventing an unambiguous identification of the components of the 160K band. However, cross-linking of cell walls previously extracted with distilled water to remove S-protein showed neither the smeared S-protein monomer nor the high molecular weight species (Fig. 5-8; lane 8) indicating the latter species contained S-protein as the only component. If the 160K product was a result of cross-linking S-protein to an integral outer membrane protein, the complex would not be expected to enter the gel.

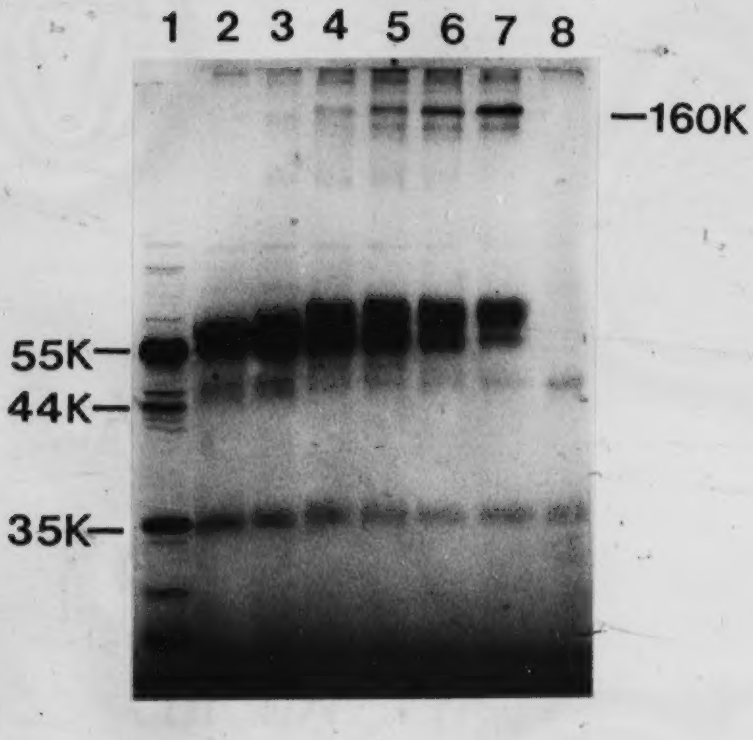

Fig. 5-8. Cross-linking of cell wall proteins with dimethyl suberimate (DMS) analyzed by SDS-PAGE. Concentrations of DMS in milligrams per millilitre : 0 (lane 1);1 (lane 2); 2, (lane 3); 5, (lane 4); 10, (lane 5); 25, (lane 6) ; 50, (lane 7). Lane 8, cross-linked distilled water washed cell walls with 50 mg/ml DMS.

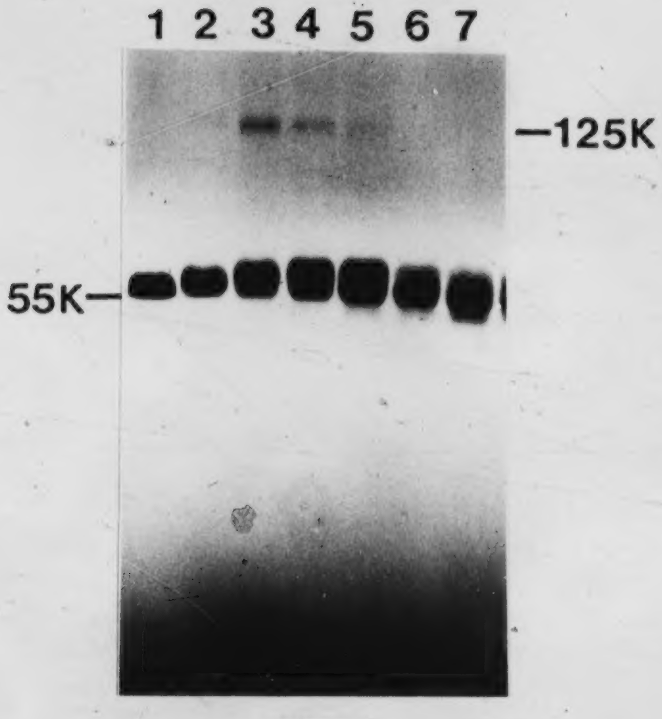

Fig. 5-9. Cross-linking of isolated S-protein multimer with dimethyl suberimate (DMS). Concentration of DMS in milligrams per millilitre: 0, (lane 1); 0.1, (lane 2); 0.2, (lane 3) ; 0.5, (lane 4); 1, (lane 5); 2, (lane 6); 5, (lane 7).

A molecular weight of 160K seemed to indicate that the major product formed was a trimer of S-protein; the cross-linked product expected in the highest abundance was a dimer of S-protein. However, because of the extensive modification of S-protein at the concentrations of DMS necessary to observe cross-linking, multimeric species were not expected to migrate at precisely integral multiples of the monomeric molecular weight. Therefore, it was unlikely that the 160K band was a trimer. The cross-linking pattern generated using cell walls could be approximated using S-protein multimer alone (Fig. 5-9). Cross-linking of free S-protein multimer with between 100-500 μg/ml DMS also produced a modification of S-protein seen in the smearing effect in the 55-60K region of the gel as well as a single major cross-linked product (Fig. 5-9; lanes 3-5). Significantly, the molecular weight of the cross-linked product species was estimated at 125K, closer to that expected for a dimer of S-protein. This was probably due to a smaller degree of monofunctional modification as compared to S-protein treated at excessive DMS concentrations (Fig. 5-8). The smearing and product formation could be produced at lower DMS concentrations with free multimer than with S-protein associated with cell walls which suggested a difference in accessibility of lysine residues to the cross-linking reagent. Because the S-layer was exposed to excessive concentrations of Na^+ at high levels of DMS, which may have perturbed its structure, a Ca^{2+}/triethanolamine buffer system was substituted in the reaction mixtures, but this did not significantly alter the results described.

The association of the S-layer with underlying components was also probed by cell surface radioiodination using Iodo-Gen (50 μg) and an ^{125}I concentration of O.5 μCi/μl. At these reagent concentrations S-protein is essentially invisible (Bingle *et al.*, 1984; Chapter 2) while the underlying outer membrane proteins are labelled. When distilled water washed cells were also treated under the same conditions there was no alteration in the labelled polypeptide pattern indicating no new proteins had become exposed upon removal of the S-layer.

State of S-protein on the surface of OCa cells

An obvious explanation for the inability of S-protein to organize into a tetragonal array in the absence of Ca^{2+} was that Ca^2 was required for formation of the morphological subunit of the array. However, when S-protein was extracted from OCa outer membranes an analyzed by rate zonal centrifugation, the sedimentation profile was identical to S-protein isolated from +Ca outer membranes. This indicated that S-protein was present on the surface of OCa cells in a tetrameric form. To determine whether there were any gross conformational differences between surface localized S-protein from +Ca and OCa cells, the surface of both cell types was radioiodinated using ^{125}I and Iodo-Gen. S-protein possesses a class of tyrosine residues which are very difficult to label with ^{125}I (Bingle *et al.*, 1984; Chapter 2). It was reasoned that if lack of Ca^{2+} caused any defect in the normal folding of the protein these residues might become more accessible to labelling. However, the cell surface radioiodination patterns of +Ca and OCa cells were identical (Fig. 5-10).

The state of S-protein on the surface of OCa cells was also probed with the proteases trypsin, thermolysin and *S. aureus* V8 protease. These proteases were chosen because of their differing specificities. Generally, trypsin cleaves after basic amino acids, thermolysin after hydrophobic residues and *S. aureus* V8 protease (in phosphate buffer) after aspartic and glutamic acid (Walker and Hayes, 1983). It was thought that such classes of residues might be clustered in different regions of the molecule and the use of three different relatively specific probes might reveal a difference in the exposure of some stretches of the amino acid chain. However, no differences in the accessibility of S-protein on the surface of Ca^{2+}-sufficient or Ca^{2+}-limited cells could be demonstrated using the proteases trypsin (Fig. 5-11a), *S. aureus V8* protease (Fig. 5-11b) and thermolysin (Fig. 5-11c). Only trypsin, and to a lesser extent *S. aureus* V8 protease (at a concentration of 1 mg/ml) cleaved S-protein while surface localized; no protease at a concentration of less than 1 mg/ml produced any effect on S-protein. Although cleaved by trypsin, the fragments of S-protein apparently remained associated with the cell surface until extracted by Sarkosyl. This phenomenon has been observed for the S-protein of *Deinococcus radiodurans* (Rachel *et al.*, 1983) as well. In contrast to surface bound S-protein, all three enzymes caused extensive degradation of soluble S-protein, a dichotomy noted for other surface array proteins (Sleytr and Messner, 1983).

Metal content of cell wall fractions

Isolated multimeric S-protein and the distilled water extracted cell wall were analyzed for their Ca^{2+} and Mg^{2+} contents by atomic absorption spectroscopy (Table 5-2). Although it was expected that S-protein would have been enriched for Ca^{2+}, this was not found. The ratio of Mg^{2+} to Ca^{2+} was the same as the ratio of these ions in the growth medium. The distilled water extracted cell wall also possessed these ions in a similar ratio. A similar analysis of 0Ca cell wall fractions showed a depletion of Ca^{2+} and an enrichment of Mg^{2+}. 0Ca cell wall fractions contained 25-30% of the Ca^{2+} present in +Ca cell wall fractions.

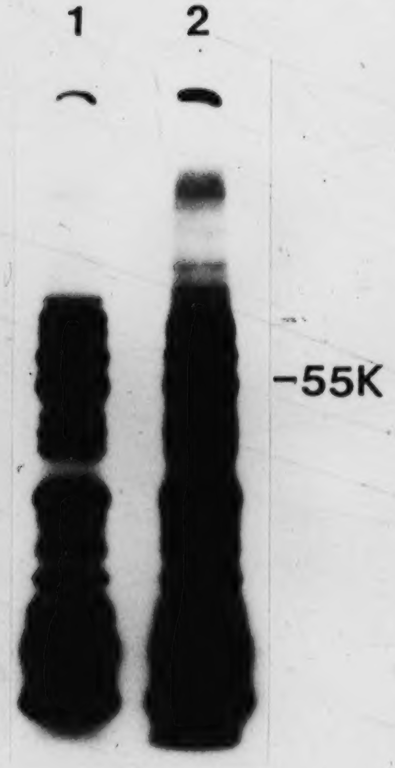

Fig. 5-10. SDS-PAGE of radioiodinated whole cells of *A. vinelandii* UW1 grown under Ca^{2+}-sufficient (lane 1) or Ca^{2+}-limited (lane 2) conditions.

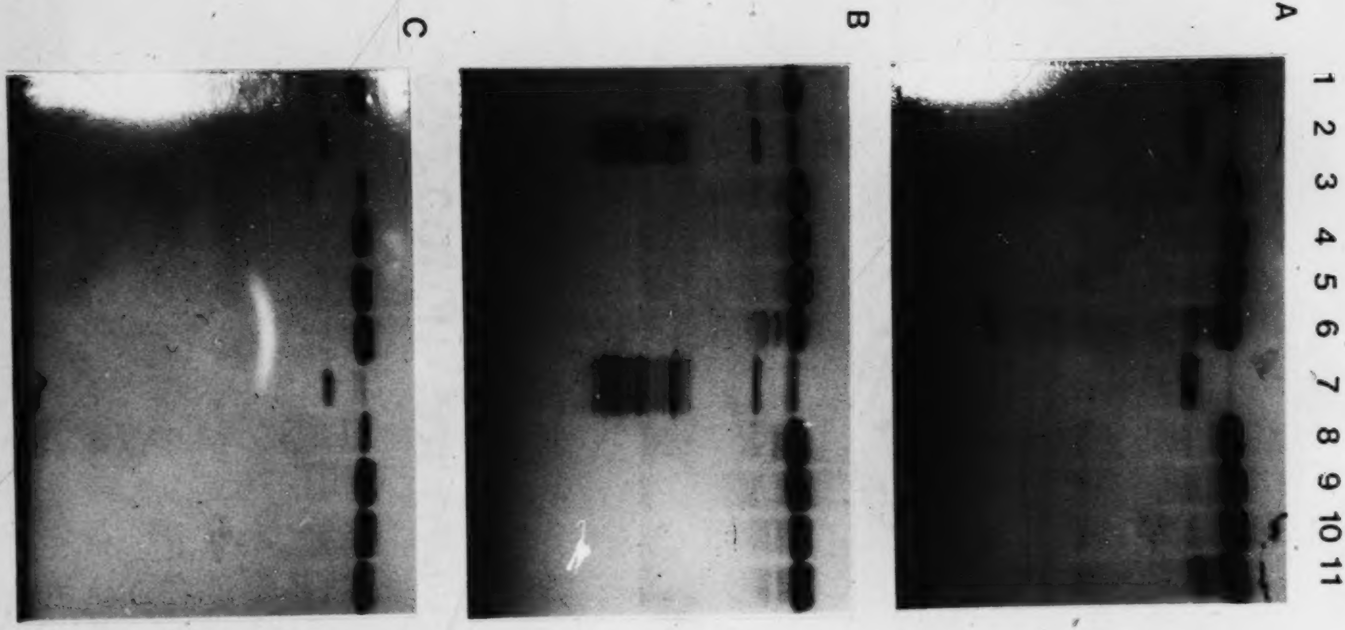

Fig. 5-11. Sensitivity of surface bound S-protein from Ca^{2+}-sufficient and Ca^{2+}-limited *A. vinelandii* UW1 whole cells to the proteases trypsin (a), *Staphylococcus aureus* V8 protease (b), thermolysin (c). S-protein standard (lane 1); S-protein extracted from outer membranes of Ca^{2+}-sufficient (lane 2) or Ca^{2+}-limited (lane 7) cells and digested in a 1:1 weight ratio with each protease; S-protein extracted from Ca^{2+}-sufficient (lanes 3-6) or Ca^{2+}-limited (lanes 8-11) cells following protease treatment. The following concentrations of each protease were employed: 0 µg/ml (lanes 3 and 8); 10 µg/ml (lanes 4 and 9); 100 µg/ml (lanes 5 and 10); 1 mg/ml (lanes 6 and 11).

Table 5-2. Calcium and magnesium content of cell wall fractions from *A. vinelandii* UW1.

Cell wall fraction		Calcium (% dry wt.)	Magnesium (% dry wt.)
S-protein	+Ca	0.276	0.224
	OCa	0.086	0.453
Cell wall[1]	+Ca	0.398	0.320
	OCa	0.095	0.635

[1] Residual cell wall following removal of S-layer by distilled water extraction

Quantitatively, the S-protein multimeric subunits accounted for 3% of the total Ca^{2+} in the cell wall and 4% of the total Mg^{2+}. Whether this represents the true distribution of these ions between the surface layer subunits and the outer membrane is unknown. It is obvious that in order to reassemble the subunits on to the cell wall *in vitro* additional divalent cations are required (Bingle *et al.*, 1986; Chapter 3) which presumably replace those extracted by the distilled water. Unlike the S-layer subunits and the outer membrane, the distilled water extract from which the subunits were concentrated was not analyzed by atomic absorption spectroscopy for Ca^{2+} and Mg^{2+}. When La^{3+} was added to the concentrated extract a precipitate resulted which made analysis impossible. While the distilled water extract undoubtedly contained additional Ca^{2+} and Mg^{2+} and its analysis would have been preferable for completeness, it is unlikely this analysis would have been really useful. It would have been impossible to specifically assign any Ca^{2+} or Mg^{2+} present to either of the two cell wall fractions.

Cationic substitution

In a final effort to confirm a specific role for Ca^{2+} in the *A. vinelandii* S-layer, whole cells were washed five times with 200 mM $MgCl_2$/10 mM Tris/HCl, pH 8.4. This treatment has been shown to displace LPS from the outer membrane of *Pseudomonas aeruginosa* (Cheng *et al.*, 1970) due to saturation of anionic sites with Mg^{2+} thereby causing disruption of the cross-linking between LPS molecules. Similarly, S-layers dependent on specific divalent cations can also be disrupted with competing cations (Beveridge and Murray, 1976b). However, this treatment did not cause disruption of the array nor did washing with 200 mM $CaCl_2$. Interestingly these treatments were found to be most effective in removing overlying masking material from the cells thereby providing excellent views of the S-layer by freeze-etch electron microscopy (Fig. 5-12). Doran (1983) reported that washing iron-limited *A. vinelandii* with 200 mM Mg^{2+} caused a "linearization" of the tetragonal array ; this result could not be repeated.

5.4 Discussion

One confusing aspect of *Azotobacter* growth requirements has been raised by this study. *A. vinelandii* strains O and OP (the parent strains of UW) have been reported to possess a Ca^{2+} requirement for optimal growth. In 1957, Norris and Jensen reported a

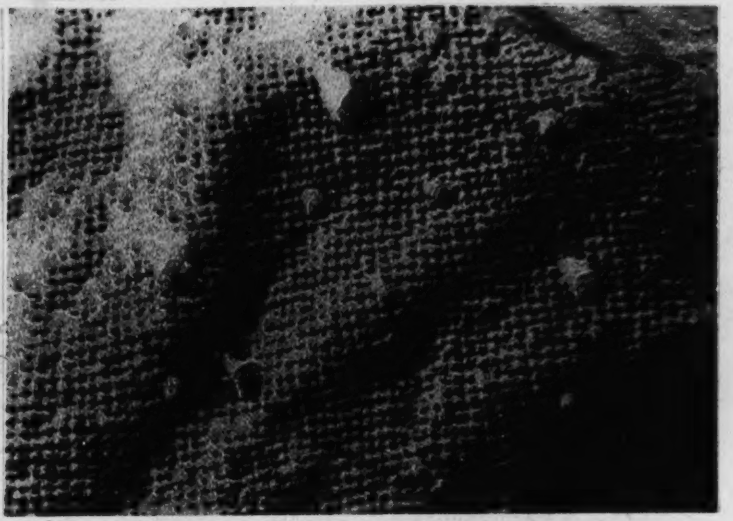

Fig. 5-12. Effect of 200 mM $MgCl_2$-10 mM Tris/HCl, pH 8.4 on the organization of the *A. vinelandii* UW1 S-layer.

requirement for 0.25 mM Ca^{2+}, but by 1977 this requirement had dropped nearly an order of magnitude to 50 μM (Barnes *et al.*, 1977, citing unpublished data). However, the media employed in this study certainly did not contain 50 μM Ca^{2+} and the cultures grew well. At present there is no explanation for the apparent differences in Ca^{2+} requirements between these strains. In conjunction with this fact, S-protein and the cell wall proper still contained a significant level of Ca^{2+}, even after many generations of growth in OCa medium. Considering the negligible levels of Ca^{2+} in the growth medium a much more severe depletion of Ca^{2+} was expected. This suggested that perhaps more Ca^{2+} was present in the growth medium than was indicated by atomic absorption spectroscopy. However, the above unexpected observations may be explained if the S-layer serves as an cation sink (Beveridge, 1979), moderating the depletion of Ca^{2+} during growth in Ca^{2+}-limited medium and masking any functional Ca^{2+} enrichment over Mg^{2+} when grown in the presence of both cations. This hypothesis seems likely in light of the findings that the S-layer has been shown to bind substantial amounts of ferric iron (Page and Huyer, 1984) and may serve as an reservoir of iron for *A. vinelandii*. Koval and Murray (1985) noted that when grown in a Ca^{2+}-deficient medium, *A. serpens* VHA could scavenge the trace amounts of Ca^{2+} present in the medium and assemble some patches of its S-layer into a regular array. *A. vinelandii* OCa medium probably contains on the order of at least 1-2 μm Ca^{2+}. Assuming the inoculum to be Ca^{2+} free (which it would not be), if the cell wall was able to scavenge this trace Ca^{2+}, it could account for 15-30% of the Ca^{2+} detected in the cell wall of Ca^{2+}-limited cells. Thus, even levels of Ca^{2+} which would be generously described as trace amounts, in comparison to that normally found in +Ca medium, could significantly inflate the experimentally determined values in the case of the OCa cell wall fractions. The residual sensitivity of OCa cells to EGTA also argues for the presence of Ca^{2+} in the cell wall, although EGTA does possess an affinity for Mg^{2+} which is 3-4 orders of magnitude lower than its affinity for Ca^{2+} (Boggis *et al.*, 1979). This leaves open the question as to whether the formation of the tetrameric unit, and the subsequent crystallization of these units into the surface layer, both require Ca^{2+}. Although it was shown previously (Bingle *et al.*, 1987; Chapter 4) that Ca^{2+} or Mg^{2+} could apparently stabilize the tetramer, the biological significance of this stabilization can, and will be questioned later in this thesis.

Although the S-layer of *A. vinelandii* requires Ca^{2+} for assembly, the ratio of Ca^{2+} and Mg^{2+} bound to the protein is the same as the ratio of these ions in the growth medium. This is a quite different result than that obtained by Beveridge (1979) for the S-layer of *Sporosarcina ureae*. The array of this organism, in contrast to *A. vinelandii*, apparently requires Mg^{2+}. Elemental analysis of surface array preparations revealed only Mg^{2+} and no measurable quantities of other cations. However, this is probably due to the fact that the array preparations were washed in 1 mM $MgCl_2$ before analysis.

Incubation of isolated S-protein with a cell wall template and either Ca^{2+} or Mg^{2+} supports its reassembly into a regular array. However, it appears that such an approach is at least partially artifactual because reassembly promoted by either cation does not return the S-layer to its native state. Buckmire and Murray (1976) reported similar findings for the Mg^{2+} reassembled S-layer of *A. serpens* VHA, but Ca^{2+}-mediated reassembly returned this surface layer to its original state and appears to correspond to an absolute requirement for Ca^{2+} for S-layer assembly in this organism (Koval and Murray, 1985). Like *A. vinelandii*, *A. serpens* VHA exports S-protein to the cell surface in Ca^{2+}-deficient medium which subsequently reassembles when the culture is presented with Ca^{2+}. However for *A. serpens*, the exported surface protein seems much more loosely bound to the cell surface being susceptible to loss into the culture fluids and by buffer washing.

While the inability of Mg^{2+}-mediated *in vitro* reassembly to return the *A. vinelandii* S-layer to its native state may not be surprising, the inability of Ca^{+2} to do so is. This is because when the reassembly is performed with S-protein surface localized *in vivo*, Ca^{2+} will organize the protein into a regular array. This suggests that S-protein exported *in vivo* achieves an association with the outer membrane which cannot be duplicated through the *in vitro* reassembly process. The nature of the outer membrane association remains obscure at this point although association with an outer membrane protein appears unlikely because cross-linking and radioiodination experiments did not reveal any major close association between S-protein and another outer membrane protein. In fact, the outer membrane appears to be cross-linked out from under the S-layer. The major cross-linking appears to take place between individual S-protein molecules. The lack of cross-linking does not appear to be due to a lack of accessible lysine residues since severe monofunctional modification of S-protein was evident, although it is possible reactive residues were not suitably disposed for cross-link formation. The S-protein of *A. serpens* VHA also exhibits little tendency to cross-link to other proteins using the reagents employed in this study (Koval and Murray, 1981).

S-protein exported to the cell surface *in vivo* clearly selects Ca^{2+} over Mg^{2+} in the *in vivo* assembly process. This apparently occurs at the level of organizing preformed morphological subunits into the regular array because distilled water extraction of cell walls from Ca^{2+}-limited cells shows that S-protein is organized into tetramers. It should be emphasized that reassembly both *in vivo* using 0Ca cells and *in vitro* using isolated S-protein and distilled water washed cells, has only been demonstrated with preformed tetramers of S-protein in *A. vinelandii.* The actual mechanism of regular array formation from unorganized S-protein is a matter of speculation. Koval and Murray (1984) speculated that for *A. serpens* VHA, Ca^{2+} was required to induce a conformational change in the S-protein to allow assembly (crystallization) of the array. However the probes of the gross conformation of surface localized S-protein of *A. vinelandii* in this study did not reveal any differences between Ca^{2+}-limited and Ca^{2+}-sufficient cells. A reversible transition between organized and unorganized surface bound S-protein has been found for the S-layer proteins of *A. serpens* VHA (Koval and Murray, 1983), *Bacillus sphaericus* 9602 (Hastie and Brinton, 1979), and *Clostridium thermosaccharolyticum* (Sleytr and Glauert, 1976). In these cases the regular pattern was destroyed by acidification and could be restored by raising the pH. However, it is likely that in contrast to Ca^{2+}-limited growth of *A. vinelandii,* the reduction in pH causes considerable alteration to the folding of the protein (Baumeister *et al.*, 1982).

This study has confirmed that the S-layer of *A. vinelandii* now joins those of the aquaspirilla in absolutely requiring Ca^{2+} for S-layer crystallization. It also points to the necessity of testing whether cation-mediated *in vitro* reassembly experiments using isolated S-proteins are really giving any biologically meaningful information on their own. The S-layer of *Acinetobacter* 199A has been reported to possess curious ionic requirements for reassembly (Thorne *et al.*, 1975) which has lead to some confusion in the literature. Beveridge (1981) stated that Mg^{2+} was required for reassembly while Koval and Murray (1984) reported Cl^- was required. The latter authors more correctly state the case. Either Na^+, Mg^{2+} or Ca^{2+} will support *in vitro* reassembly of this S-layer as long as the companion anion is Cl^-. Interestingly, the Cl^- requirement is negated if a cell wall template is provided and monovalent cations are no longer competent to support reassembly. These facts seem rather surprising, and suggest as with *A. vinelandii*, that the *in vitro* reassembly process is not providing correct information on the *in vivo* assembly process. The stability of reassembled surface arrays of *Acinetobacter* 199A need to be examined to pin down the real ionic requirement. If the results with *A. vinelandii* and *A. serpens* VHA are any indication, the *Acinetobacter* S-layer may also possess a specific Ca^{2+} requirement.

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6. General Discussion

The tetragonal surface layer of *Azotobacter vinelandii* possesses certain similarities to both of the other well characterized tetragonal S-layers from the gram-negative bacteria *Acinetobacter* 199A and *Aeromonas salmonicida*. This may indicate some common principles of construction like those observed for the tetragonal S-layers of the gram-positive spore-forming bacteria (Burley and Murray, 1983). The molecular weight of the single proteins constituting each layer are similar, 62,000 for *Acinetobacter* 199A, 50,000 for *A. salmonicida* and 60,000 for *A. vinelandii*. The fine structure of the *A. salmonicida* S-layer determined by Stewart *et al.* (1986) is remarkably similar to that determined for *A. vinelandii*, and the divalent cation-mediated interactions of the protein constituting the *Acinetobacter* 199A layer are comparable to those of the *A. vinelandii* S-protein.

Although the fine structure of the *A. salmonicida* S-layer is similar to that of *A. vinelandii*, Stewart *et al.* (1986) reported that the S-layer of *A. salmonicida* could exist in two configurations (Type I and Type II); *A. vinelandii* possesses a counterpart of the Type II layer. If the Type I layer existed for *A. vinelandii*, it (see Fig. 4-1C) would show the dominant tetrad units alternating with the linking units, except the latter structure would not be rotated and the delicate linkers extending from the tetrads to the linking unit proper would be absent. In other words, the layer would appear to be composed of two different alternating subunits since there would apparently be no connectivity between them. Stewart *et al.* (1986) postulated that the two configurations of the layer were interconvertable, in this way the cell could control its permeability because there would be larger holes between the subunits of the Type I layer (the one lacking the connections between the two types of subunits). How this conformational change could be achieved was not discussed, but an analogy was drawn between these findings and the regularly arranged proteins of the eukaryotic gap junction which exist in a hexagonal lattice like certain bacterial S-layers. Uwin and Ennis (1984) showed that the hexameric units of the gap junction protein array could undergo a very slight conformational change, which caused the holes between the subunits to increase or decrease in size, thus mediating a permeability change. Interestingly, this conformational change was triggered by Ca^{2+}. Whether the interpretation of the two types of S-layer pattern seen on *A. salmonicida* cells by Stewart *et al.* (1986) turns out to be correct, is due to a preparation artifact, or any number of other reasons, will have to await further experimentation. However, if their analogy to the gap junction protein array is a valid one, it may help to explain the Ca^{2+}

induced crystallization of the *A. vinelandii* S-layer. Although cells grown in the absence of Ca^{2+} possessed no visible S-layer, the surface localized S-protein tetramers present on Ca^{2+}-limited cells did not possess any gross conformational differences compared to those found on Ca^{2+}-sufficient cells. Nevertheless, Ca^{2+} caused crystallization of the subunits into a tetragonal array similar to the Ca^{2+} induced crystallization of the gap junction protein array into its alternative configuration.

Ca^{2+} would be a good candidate as a trigger for S-layer crystallization. It is usually stated that bacteria actively maintain the intracellular concentration of Ca^{2+} below that of the environment for reasons such as prevention of intracellular phosphate precipitation. *A. vinelandii* possesses a calcium/proton antiporter which presumably serves this function (Zimniak and Barnes, 1980). Interestingly, *A. vinelandii* also possesses a Ca^{2+} uniporter to catalyze Ca^{2+} entry into the cell, presumably for some as of yet unidentified Ca^{2+} regulated intracellular process. One would expect that S-layer crystallization should be prevented until the subunits have made their way to the outside of the cell. This does not happen with S-layers from gram-positive organisms which can be found on the inside as well as the outside of the peptidoglycan layer (see Sleytr and Glauert, 1976). One would therefore expect that the cell has little control of the construction of the S-layer outside the cytoplasmic membrane and even less control outside the outer membrane and would require an environmental trigger for array assembly. This trigger could be an elevated Ca^{2+} concentration. There are two other explanations which come to mind which would require more effort on the cell's part: (1) cleavage of the N-terminal signal sequence from the precursor S-protein during(?) export renders the mature form capable of crystallization or (2) the protein is enzymatically modified outside the cell so it can crystallize. There may be a precedent for this latter suggestion in *A. vinelandii*. This organism secretes a capsule of mannuronic acid which is epimerized into guluronic acid extracellularly, by a Ca^{2+} requiring extracellular enzyme (Haug and Larsen, 1971). While the above explanations are possible, they are not likely because there is obviously an additional requirement for crystallization in Ca^{2+}-limited cells.

The *A. vinelandii* S-protein as well as other S-proteins possess a defined secondary structure in solution which includes a significant level of ß-sheet structure. The functional significance of this structure is obscure at this point, but it is interesting that other bacterial outer membrane proteins (porins) also possess ß-rich secondary structures and no detectable α-helix by circular dichroism. Porins were once classed as surface layers (Sleytr, 1978) due to their ability to form regular hexagonal lattices *in vitro* first described by Rosenbusch (1974). A controversy ensued as to whether such regular arrays were also formed *in vivo* (see Lugtenberg and van Alphen, 1983) but it appears that this idea has lost favour. The similarity between porins and regular surface array proteins in these respects is interesting. S-layer proteins are thought to serve some permeability function and are thought in evolutionary terms to be the most primitive membranes. Perhaps these proteins formed the basis for the porins of gram-negative bacteria. The spirochete cell wall may be a good example of how the distinction between porins and S-layers is blurred. As mentioned previously, spirochetes are thought to possess an S-layer but it appears to be located within the outer sheath. Unlike the situation with porins, there is no question that the regular structure is present *in vivo* (Masuda and Kawata, 1982) and the regular array can even be dissociated and reassembled *in vitro* with Mg^{2+}. Another extensively studied protein which is similar to the *A. vinelandii* S-protein in its amino acid composition and ß-rich secondary structure is that which forms a nonregularly structured layer on the surface of the spore of *Myxococcus xanthus*. The following comparison between the behaviour of the *M. xanthus* spore coat protein, bacterial porin proteins and the *A. vinelandii* S-protein is useful.

Monomeric S-protein from *A. vinelandii* does not reassemble onto the surface of distilled water washed cells yet the tetrameric form is competent to do so. However, no major alteration in the secondary structure between the two quaternary forms of S-protein is detected by circular dichroism. The lack of a detectable conformational difference between monomeric and oligomeric porin from *Salmonella typhimurium* was also demonstrated by Tokunaga *et al.* (1979) so oligomerization does not necessarily involve a conformational change. Like the *A. vinelandii* S-protein, which is only active in reassembly in the oligomeric state, the porin from *S. typhimurium* is only active in the trimeric, not the monomeric state. Similarly the surface protein of *M. xanthus* does not undergo any conformational change in response to Ca^{2+} although this cation is required for its binding and reassembly onto to the spore surface (Inouye *et al.*, 1979, 1981). Wistow *et al.* (1985) do not consider it unreasonable that this protein may be cross-linked via Ca^{2+} to receptors on the spore surface, or to other protein molecules of the surface layer, through surface Ca^{2+} sites which do not affect the conformation of the molecule. A similar situation could exist with the *A. vinelandii* S-protein.

The tetrameric form of S-protein which apparently constitutes the morphological subunit of the array can be stabilized with divalent cations yet the monomer cannot be induced to reform the tetramer under the same conditions. The results obtained with cells grown under Ca^{2+}-limitation seems to suggest that the association of the tetramer with an outer membrane component, achieved *in vivo*, provides strong attachment of the subunits to the outer membrane and creates a selectivity for Ca^{2+} over Mg^{+2} during the assembly of the regular array. The component is apparently not an outer membrane protein and it is interesting to speculate that this component might be lipopolysaccharide (LPS). Evidence exists for an association between the *A. salmonicida* S-layer and the O-side chains of LPS (Belland and Trust, 1985) and synthesis of LPS is apparently linked to S-layer assembly in *Acinetobacter* 199A (Thorne *et al.*, 1976). Labischinski *et al.* (1985) in a recent study of the conformation of LPS, indicated that the O-side chains were not present in an extended conformation but were coiled. If reassembly is performed *in vitro* with preformed subunits, the LPS molecules may prevent correct access of the subunits to their normal position which would not be a problem if assembly of the S-layer was performed from the inside-out, i.e., as it occurs *in vivo*. If the assembly of the tetramer, in conjunction with the O-side chains of LPS is vectorial, perhaps once the tetramer has dissociated, its assembly pathway can not be repeated *in vitro* from the opposite direction. Schenk (1978) provides an interesting piece of data that may support this idea. Using the encapsulated *A. vinelandii* strain 12837 he found that much carbohydrate was released from whole cells when the surface protein was removed by distilled water washing. Analysis of the carbohydrate component showed the sugars were not derived from capsule but from the O-side chains of the LPS, yet surprisingly no lipid A was found in the preparation. In the present study, no significant level of LPS could be found in S-protein preparations from the nonencapsulated strain *A. vinelandii* UW1 but the observations of Schenk (1978) deserve further investigation as does the association of the S-layer with LPS. The association of the S-protein subunits with a diffusable outer membrane component such as LPS, could aid in the crystallization of the S-layer (Uzgiris and Kornberg, 1983).

If association with an outer membrane component, perhaps LPS, is necessary for tetramer formation and stability, how do divalent cations act by stabilizing the tetramer? Although the notion is quite unsettling, it must be considered that divalent cations may artificially stabilize the tetramer and this stabilization has no biological significance. A critical experiment testing whether the divalent-cation stabilized tetramers were still biologically active in *in vitro* reassembly could not be performed. This was due to the fact that while the cations stabilized the tetramer they also caused aggregation of S-protein making it impossible to use in reassembly studies. In order to prevent aggregation, the S-protein-cation mixtures had to be diluted to less than 40 μg/ml protein, which was too dilute a solution to use in reassembly assays. Secondly, when an attempt was made to concentrate the dilute S-protein-cation mixtures by ultrafiltration, the protein aggregated and formed a membrane-like film on the ultrafiltration membrane, almost preventing further passage of the ultrafiltrate through the membrane. Although the possibility that divalent cations may artificially stabilize the tetramer might seem quite a radical interpretation of the data, this question is also raised by the apparent artificiality of the *in vitro* reassembly studies of the S-layer. These considerations also lead to a questioning of the utility of studying ion requirements of S-proteins from *in vitro* studies. This problem is addressed below.

It was mentioned earlier that a major difference between S-layers from gram-positive and gram-negative organisms was their stability to EDTA or EGTA. This appear related to the fact the S-layers from gram-negative organisms usually possess a divalent cation requirement while S-layers from gram-positive species do not. However, there appears to be two exceptions to this generalization, the surface arrays of *Sporosarcina ureae* (Beveridge, 1979) and *Bacillus brevis* 47 (Yamada *et al.*, 1981). Both of these S-layers have been reported to possess a Mg^{2+} requirement for assembly into a regular array. An examination of these two proteins illustrates the problem of trying to elucidate ion requirements of these proteins *in vitro*. Beveridge (1979) found that if the surface array of *S. ureae* was isolated free from the cell wall in the presence of 1 mM Mg^{2+}, it could subsequently be disrupted by EDTA. Mg^{2+} also prevented the disruptive effects of other divalent cations (Ca^{2+} and Sr^{+2}) on S-layer organization *in vitro*. He concluded that the array required Mg^{2+} for its structure but this conclusion is not warranted. The stability of the native array to EDTA was never tested, i.e., an array not first stabilized with Mg^{2+} was not tested for sensitivity to EDTA. It is not surprising that if Mg^{2+} is needed to stabilize the array, that subsequent treatment with EDTA will disrupt it. The argument could be made if Mg^{2+} was required to maintain the integrity of the array in the first place, a divalent cation of some sort and probably Mg^{2+} is required *in vivo*. However, this is not necessarily the case. For example, a large organic cation like Tris can be used to reassemble the *Acinetobacter* 199A array *in vitro* although it apparently requires a divalent metal ion *in vivo* (Glauert and Thornley, 1973; Thornley *et al.*, 1974). Similarly preliminary results with the *A. vinelandii* S-protein also suggest Tris can produce reassembly. Secondly, it has long been known for LPS which is cross-linked *in vivo* by Mg^{2+} and Ca^{2+}, that organic cations like Tris, and polycations like polyamines, can interact with the same sites as Mg^{2+} and Ca^{2+} (Hancock, 1984). Since there is no information on the effect of EDTA on the native array of *S. ureae* and no information on its association with underlying cell wall layers the conclusion that Mg^{+2} is required for this array is premature especially considering the ability of the cell wall to stabilize associated surface arrays in gram-positive organisms (Hastie and Brinton, 1979; Sleytr, 1976) and the documented problems in determining *in vivo* ion requirements from *in vitro* characteristics.

The second example of an S-protein from a gram-positive organism that apparently requires Mg^{2+} for S-layer assembly is the double S-layer of *B. brevis* 47 (Yamada *et al.*, 1981). A peculiarity of this organism which is rarely considered is that it hypersecretes its two cell wall proteins into the growth medium to levels of 10-12 mg protein/ml. This secretion is not a consequence of this type of cell wall since the closely related *B. brevis* S1 S-layer is assembled without concomitant secretion of vast quantities of protein (Abe *et al.*, 1983). In fact, the secretion of the cell wall by the soil isolate *B. brevis* 47 seems an odd growth strategy from an energetic point of view, considering that soil is an extremely oligotrophic environment. The obvious conclusion regarding this organism is that it is a mutant which has a defect in assembling its surface layer. Specifically, it has a problem knowing when its surface is covered and continues to make cell wall proteins even though they are no longer needed. All other S-layer proteins examined have their synthesis tightly controlled and produce only as much as is necessary or slightly more (see Koval and Murray, 1985). In fact, the energetic drain as a consequence of S-layer production is thought to be so significant, it is frequently stated that spontaneous mutants lacking S-layers outgrow the wild-type leading to laboratory cultures which lose their S-layers. (Sleytr and Messner, 1983). This does not always happen, just as loss of capsule during serial culture is not a certainty. An alternative to this explanation, is that the S-layer receptor site in the cell wall may have a defect so S-protein is continually shed into the medium. This could be analogous to the situation with rough mutants of *A. salmonicida* which cannot produce the O-side chains of the LPS causing the loss of the S-layer into the medium (Belland and Trust, 1985). Whatever the explanation, it is highly likely that the *B. brevis* 47 is atypical.

The conclusion that the S-layer of *B. brevis* 47 requires Mg^{2+} comes from two experiments. The first is that if Mg^{2+} is added to the medium at 5-10 mM inhibition of cell wall shedding results. In fact, Ca^{2+} will work as well. Again the argument can be made that surely if Mg^{2+} or Ca^{2+} prevent cell wall shedding, these ions must serve this function *in vivo*. Unfortunately this conclusion cannot be made. The correction of a structural defect in the cell wall by Mg^{2+} and Ca^{2+} has been documented many times in gram-negative organisms (Hancock, 1984) for a number of different mutations. Even in the case where many outer membrane proteins (including porins and lipoprotein) are missing, leading to cell lysis, a low concentration of Ca^{2+} or Mg^{2+} can restore cell integrity (Sonntag *et al.*, 1978). An hypothesis for how divalent cations can substitute for integral outer membrane protein molecules is difficult to come by. Nevertheless, these experiments clearly demonstrate that simply because a divalent cation apparently corrects a structural defect in the cell wall, this does not say anything about the physiological significance of the treatment. The same can be said for the divalent cation-mediated inhibition of S-layer loss in *B. brevis* 47. The second line of evidence that divalent cations are important to the S-layer of *B. brevis* 47 is that if the cell wall is treated with EDTA the S-layer is released. But Yamada *et al.* (1981) isolated cell walls from *B. brevis* 47 in the same way Beveridge (1979) did for *S. ureae*. The cell wall S-layer:interaction was first stabilized with Mg^{2+} then treated with EDTA. Again, it is not surprising, and perhaps expected, if you first stabilize with Mg^{2+}, and then treat with an agent which removes Mg^{2+}, that destruction of the S-layer results.

The detailed examination of the experiments described above indicates there is little good evidence for divalent cation involvement in the S-layers of any of the gram-positive organisms studied so far. Secondly, these experiments and those reported in this thesis with *in vitro* assembly of the *A. vinelandii* S-layer, point out the highly suspect nature of the information derived from such experiments with respect to establishing ionic requirements for *in vivo* assembly. S-layer research has taken advantage of the ability of these proteins to self-assemble *in vitro* without due regard to the problem of the biological significance of some of the results and the possibility of artifacts. An analogy can be drawn to reconstituted biological membranes. It is usually stated or inferred that biological membranes, especially the lipid components, are a self-assembling structure. However, native membranes exhibit asymmetry in the phospholipid distribution between the two leaflets of the bilayer and in protein topology. Yet when the phospholipids are extracted and mixed with the integral membrane proteins a symmetrical membrane usually results (Reithmeier, 1985). Although the system has self-assembled and would appear physically as a bilayer, it has not returned to the native state.

S-layers do not exist in solution, they exist intimately associated with underlying cell wall layers which influence their stability. One should no more consider working with an S-layer protein in solution without regard to its association with the rest of the cell wall than one would work with an integral membrane protein in the absence of a detergent or phospholipid bilayer. This does not mean that useful information cannot be derived from *in vitro* studies (see Kist and Murray, 1984) but due regard should be given to whether the results are biologically meaningful. This was the approach first used by Buckmire and Murray (1976), but unfortunately, not by many others. The use of mutants defective in aspects of S-layer assembly (Belland and Trust, 1985) and where possible, growth conditions which perturb the *in vivo* assembly of S-layers (Doran *et al.*, 1987, Chapter 5; Beveridge and Murray, 1976; Koval and Murray, 1985) should greatly enhance the determination of meaningful properties of bacterial surface arrays.

6.2 References

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